YAP regulates post-fracture periosteal expansion through both cell intrinsic and extrinsic co-transcriptional programs.

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Introduction: The periosteum comprises a pool of quiescent progenitor cells that proliferate and mobilize upon fracture to initiate repair. Osterix-expressing osteoblast precursors proliferate in the early stages, and their transition from a quiescent to proliferative state is transcriptionally regulated. We previously found that the transcriptional regulators, YAP and TAZ, are required for periosteal cell proliferation after fracture[1]. YAP and TAZ lack DNA-binding motifs but bind to and regulate the activity of other transcription factors (co-effectors) to control gene expression. However, we do not know the identities of the target genes or the transcriptional co-effectors by which YAP and TAZ control periosteal expansion during fracture repair. The goals of this study were to define the early transcriptional targets of fracture-induced YAP activation in periosteal cells and to identify putative transcriptional co-effectors through analysis of YAP-induced chromatin accessibility by ATAC-Sequencing.

Materials and Methods: Here, we used an intramedullary pin-stabilized femoral osteotomy model in both wild type and Osterix-conditional YAP/TAZ knockout mice (Osx-Cre:GFP; $YAP^{fl/fl}$;$TAZ^{fl/fl}$). First, we mapped fracture-induced YAP activation in periosteal progenitor cells by YAP and osterix immunostaining in wild type periosteum at four days post fracture (4 DPF). Next, we evaluated proliferation in wild type and Osx-conditional YAP/TAZ knockout mice by injecting animals with 10mg/kg 5-ethynyl-2'-deoxyuridine (EdU) 3 hours before sacrifice. To model fracture-induced YAP activation *in vitro*, we isolated periosteal cells from fracture-activated periostea in mice expressing a doxycycline (Dox)-inducible mutant YAP transgene, YAP^{S127A}, which escapes degradation by the Hippo pathway (CMV-Cre; R26R-rtTA$^{fl/fl}$; tetO-YAP^{S127A}). We treated cells with 1uM dox for 8 hours, which we demonstrated previously to coincide with the earliest detectable YAP^{S127A}-induced target gene expression. We performed bulk mRNA sequencing (RNA-seq) and assay for transposase accessible chromatin with sequencing (ATAC-seq) in both wild type and YAP^{S127A} cells with or without Dox. We scanned chromatin that was preferentially opened in YAP^{S127A}-expressing cells for DNA-binding motifs of known transcription factors (transcription factor footprints). This allowed us to identify putative YAP binding partners, and subsequently confirm their ability to bind to YAP in periosteal cells by co-immunoprecipitation, followed by western blots. These orthogonal experiments allowed us to identify *BMP4* as a YAP target gene in periosteal cells. Finally, we injected BMP4 into the fracture gap for the first four days post fracture to assess its role in early periosteal expansion.

Results: Fracture induces rapid periosteal expansion, characterized by activation and proliferation of both chondroprogenitor and osteoprogenitor cells. Osx-conditional deletion of both YAP and TAZ significantly reduced periosteal cell proliferation and periosteal expansion (Fig 1A, B). YAP^{S127A} activation in periosteal osteoblast precursors cultured *in vitro* induced activation and suppression of diverse genes. Quantitative gene set variation analysis revealed ~600 significantly differentially regulated gene sets after YAP activation (Fig 1C). We focused on 236 gene sets most relevant to progenitor pool expansion during fracture repair. Analysis of DNA motifs present in differentially accessible chromatin revealed chromatin loci containing the consensus binding motifs of the TEAD and Smad2/3 transcription factors (Fig 1D). Consistently, YAP^{S127A} activation elevated canonical YAP-TEAD complex target genes (CTGF, Cyr61, Ankrd1) and enriched Smad2/3-target gene sets, supporting roles for both TEAD and SMAD2/3 as putative transcriptional co-effectors of YAP in periosteal osteoblast precursors. TEAD co-immunoprecipitated with YAP in periosteal cells, confirming physical YAP-TEAD interaction (Fig 1D). From our RNA-seq analysis, we found that YAP promoted cell proliferation and periosteal expansion via cell intrinsic mechanisms, including cell cycle progression, and promoted transcription of secreted factors that signal to other cells such as Cyr61, CTGF, BMP4 and interleukins. This was confirmed by our *in vivo* analysis in Osx-conditional YAP/TAZ knockout mice, where the number of both Osx^+ and Osx^- cells in the periosteum was significantly reduced (Fig 1B). This orthogonal approach using mRNA- and ATAC-Sequencing and gain- and loss-of-function models allowed us to identify and validate broad transcriptional programs regulated by YAP/TAZ activation shortly after fracture. Notably, we identified BMP4 as a robust YAP target gene. To evaluate the function of BMP4 in YAP/TAZ-mediated periosteal expansion, we injected BMP4 directly into the fracture gap for the first 4 days post-fracture. BMP4 treatment rescued the periosteal expansion defect caused by YAP/TAZ deletion (Fig 1E).

Discussion: Here, we show that YAP is activated in Osx-expressing osteoblast precursor cells shortly after fracture to initiate periosteal cell proliferation, survival, and periosteal expansion. Non-biased ATAC- and mRNA-seq reveal putative YAP/TAZ target gene programs and transcriptional co-effectors in isolated periosteal osteoblast precursors. YAP activation promoted cell proliferation and induced genes involved in cell-autonomous regulation of proliferation and cytoskeletal regulation, but also promoted transcription of proliferation-inducing paracrine factors, including BMP4. YAP activation also downregulated gene sets involved in apoptosis, suggesting that preservation of periosteal progenitor cell survival may also be important in the complex environment of the early post-fracture niche. This approach allowed us to identify BMP4 as a YAP target gene in periosteal cells and confirm its function in YAP/TAZ-mediated periosteal expansion during early fracture repair. Interestingly, BMP4 did not rescue the number of EdU+ proliferating cells in the periosteum, indicating that it may play a role in promoting matrix production, thereby rescuing the periosteal thickness defect caused by YAP/TAZ deletion.

Significance/Clinical Relevance: Understanding the molecular mechanisms through which YAP and TAZ mediate the transition from quiescent to proliferative periosteal cells could enhance our understanding of bone healing and guide future therapeutic strategies for impaired bone regeneration.

References:[1] Kegelman+, *JBMR* 2020 [2] Corces+, *Nat. Methods* 2017 **Acknowledgements:** This work was supported by NIH R01-AR073809.

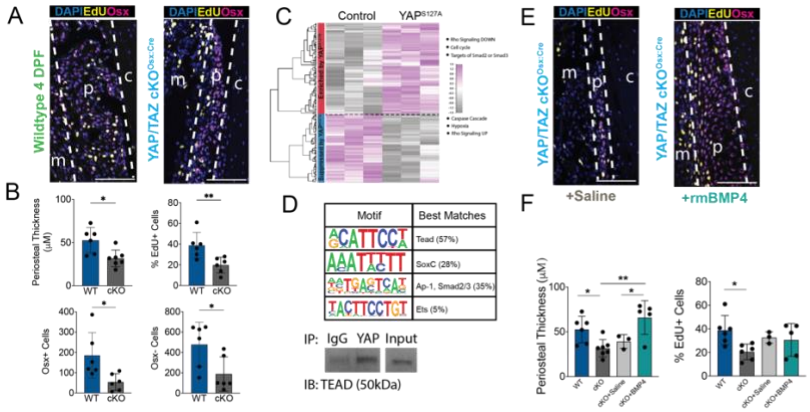

Figure 1: **A**. Osx-conditional deletion of YAP/TAZ results in inadequate periosteal expansion in early fracture repair. **B**. YAP deletion reduces the number of targeted as well as non-targeted cells in the periosteum. **C**. Bulk mRNA-Seq indicates YAP mediated changes in cell intrinsic and extrinsic programs. **D**. ATAC-Seq reveals putative YAP binding partners, of which, TEAD is confirmed by co-IP. **E**. BMP4 delivery into the fracture gap rescues the periosteal expansion defect by YAP/TAZ deletion. **F**. Quantification of cell proliferation and periosteal thickness upon BMP4 delivery.
m=muscle, p=periosteum, c=cortical bone
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