Localization of YAP activity in developing skeletal rudiments is responsive to mechanical stimulation

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November 2019

Keywords: skeletal development, morphogenesis, chondrogenesis, mechanical regulation, mechanosensory, Hippo, YAP

Grant sponsor: Irish Research Council, Wellcome Trust
ABSTRACT

Background: Normal skeletal development, in particular ossification, joint formation and shape features of condyles, depends on appropriate mechanical input from embryonic movement but it is unknown how such physical stimuli are transduced to alter gene regulation. Hippo/YAP signaling has been shown to respond to the physical environment of the cell and here we specifically investigate the YAP effector of the pathway as a potential mechano-responsive mediator in the developing limb skeleton. Results: We show spatial localization of YAP protein and of pathway target gene expression within developing skeletal rudiments where predicted biophysical stimuli patterns and shape are affected in immobilization models, coincident with the period of sensitivity to movement, but not coincident with the expression of the Hippo receptor Fat4. Furthermore, we show that under reduced mechanical stimulation, in immobile, muscle-less mouse embryos, this spatial localization is lost. In culture blocking YAP reduces chondrogenesis but the effect differs depending on the timing and/or level of YAP reduction. Conclusions: These findings implicate YAP signaling, independent of Fat4, in the transduction of mechanical signals during key stages of skeletal patterning in the developing limb, in particular endochondral ossification and shape emergence, as well as patterning of tissues at the developing synovial joint.

INTRODUCTION

As cells of the developing limb bud proliferate and differentiate, they must calibrate their gene regulatory output according to the molecular and mechanical signals they receive in order to form properly shaped, functional tissues. We and others have previously shown that normal skeletal development relies on appropriate mechanical input from embryo movement, with similar skeletal abnormalities observed in a variety of immobilization models, from muscleless mouse embryo limbs where the absence of muscle is caused by different genetic lesions (Myf5<sup>nlacZ/nlacZ</sup>/MyoD<sup>−/−</sup>, Pax3<sup>Spd/Spd</sup>), with intermediate effects seen in the case of reduced muscle mass (35-55% reduced muscle mass in Myf5<sup>nlacZ/nlacZ</sup>/MyoD<sup>−/−</sup>), to embryos with non-contractile muscle (Mdg<sup>−/−</sup>), as well as pharmacologically immobilized chick and zebrafish embryos, (reviewed in Shea et al., 2015; Rolfe et al., 2018). The resulting skeletal defects include altered rudiment morphology, especially at rudiment termini (Roddy et al., 2011; Nowlan et al., 2014; Sotiriou et al., 2019), fused and mishapen joints (Kahn et al., 2009; Nowlan et al., 2010; Roddy et al., 2011; Brunt et al., 2015) and abnormal endochondral ossification (Nowlan et al., 2008; Shwartz et al., 2012). We have previously used a comparative transcriptomics approach to show extensive alteration of gene expression in immobilized developing humeri and associated joints: Genes associated with Wnt signaling and other regulatory pathways were among the
most sensitive to immobilisation (Rolfe et al., 2014b). We recently showed that Wnt and BMP signaling pathways are reciprocally regulated at the joint interface in response to movement (Rolfe et al., 2018; Singh et al., 2018). However, precisely how cells transduce physical stimuli to impact gene expression remains unknown. Here we explore the Hippo pathway, and specifically the YAP effector, as a potential mechanotransductive mechanism in the developing limb skeleton.

Yes-Associated Protein (YAP) and Transcriptional Activator of PDZ binding motif (TAZ) are transcriptional regulators that allow cells to interpret mechanical stimuli in a way that affects gene expression and ultimately cell identity. First described as intracellular mediators of the Hippo signaling pathway (see below; (Huang et al., 2005a), YAP/TAZ have since been demonstrated to also respond to actin and microtubule cytoskeletal dynamics (Dupont et al., 2011; Zhao et al., 2012). Both the canonical Hippo pathway and the cytoskeleton are sensitive to the physical environment of the cell and control YAP/TAZ activity accordingly (Dupont et al., 2011; Halder et al., 2012).

The Hippo pathway balances cell proliferation and differentiation during mammalian development at the pre-implantation stage, where it distinguishes the undifferentiated cells of the inner cell mass from those of the placenta/trophectoderm by regulating gene expression and therefore specifying cell identity (Nishioka et al., 2009; Posfai et al., 2017). Activity of Hippo pathway components is also required for normal organogenesis of lungs, heart, kidneys, dentition, and craniofacial skeleton (Reginensi et al., 2013; Reginensi et al., 2015; Zhou et al., 2015; Fu et al., 2017; Posfai et al., 2017; Wang and Martin, 2017; Wang et al., 2017). However, while YAP activity during early lineage determination is modulated by contact inhibition, the relationship between mechanotransduction and Hippo signaling during organogenesis remains unclear. Work on mesenchymal stem cells in culture demonstrated that YAP/TAZ can act as cellular mechanotransducers. Elegant in vitro experiments showed that cell density and substrate stiffness shape actin filament architecture, thereby modulating YAP/TAZ activity and linking mechanical aspects of the extracellular environment to YAP/TAZ-mediated transcription of downstream target genes (Dupont et al., 2011; Aragona et al., 2013; Rauskolb et al., 2014). Interestingly, this effect on YAP/TAZ activity was determined to be independent of canonical Hippo signaling and was suggested to be mediated by the actin cytoskeleton (Dupont et al., 2011). However, while the role of YAP/TAZ in sensing mechanical cues has been elegantly demonstrated in vitro, a similar role in tissue differentiation in vivo has yet to be shown.

First described in Drosophila, the Hippo pathway consists of a kinase cascade which regulates the activity of co-transcriptional activators (Huang et al., 2005b). In mammals, an intracellular kinase cascade of MST1/2 and LATS1/2 mediates the phosphorylation and subcellular
localization of YAP and/or TAZ (Zhao et al., 2010). When unphosphorylated, YAP and TAZ can be translocated to the nucleus where they bind TEAD transcription factors (TEAD 1-4) to regulate target gene expression, while phosphorylated YAP is inactive (Vassilev et al., 2001; Zhao et al., 2008b). In mammals, the upstream regulation of MST1/2 is not clear: while in Drosophila the cadherin Fat (Ft) acts as a cell surface regulator, a similar role for the mammalian orthologue Fat4 (Rock et al., 2005), has been questioned (Mao et al., 2011). Further, regulation of LATS1/2 and YAP/TAZ phosphorylation outside the canonical kinase cascade has been demonstrated (Zhao et al., 2012; Meng et al., 2015), indicating that regulation of Hippo pathway components could differ between Drosophila and mammals. Numerous YAP/TAZ target genes have been identified (Zhao et al., 2008b; Zhang et al., 2009; Zanconato et al., 2015) including Cyr61 (cysteine-rich angiogenic inducer 61) and Ctgf (connective tissue growth factor), encoding matricellular proteins which have been implicated in long bone fracture healing and articular cartilage repair (reviewed in Chen and Lau, 2009).

YAP and TAZ are paralogs and while they share structural similarity and conserved binding domains, there is evidence that they are functionally distinct and can act opposingly (reviewed in Zhao et al., 2008a). YAP knockout mice are embryonic lethal at E8.5, and targeted deletion of YAP in cartilage cells results in longer bones and elevated hypertrophy (Morin-Kensicki et al., 2006; Deng et al., 2016). TAZ knockout mice live to adulthood with slightly reduced lifespans (Hossain et al., 2007) and relatively normal adult skeletons, suggesting distinct roles for YAP and TAZ and prompting a focus on YAP investigation during skeletogenesis. We found only two descriptions of YAP expression and localization in the developing skeleton, with apparent contradictory findings (Karystinou et al., 2015; Deng et al., 2016). While indicating that YAP regulation is important during skeletal development, these studies also emphasize the need for more information about the role of YAP. In particular, an important gap in our knowledge is the possibility of a link between YAP activation and the mechanical impact on skeletal development from embryo movement.

YAP represents a focal point for the balance of proliferation and differentiation and, crucially, its activity is known to respond to mechanical cues in other contexts. The indication that it plays a role in skeletogenesis opens the intriguing possibility that it could be involved in transducing the physical environment of the cells of the skeletal rudiments into gene regulatory output. As noted above, although we know the importance of mechanical stimuli to appropriate bone and joint development, the mechanism by which mechanical cues are sensed by differentiating cells and transmitted to a genetic regulatory response remains unknown. Here we examine the expression of Hippo pathway components in developing skeletal rudiments during the early period of sensitivity to movement, demonstrating that YAP protein and pathway target genes show localized patterns that correspond to regions previously shown to be altered in immobile
embryos (reviewed in Shea et al., 2015; Rolfe et al., 2018). Furthermore, examining
skeletogenesis in normal and immobile, muscle-less embryos (Splotch-delayed, $Pax3^{Spd/Spd}$
(Vogan et al., 1993) we show alterations in YAP activation under reduced mechanical
stimulation.

**RESULTS**

Hippo pathway components are highly expressed in the developing humerus during the
period of early sensitivity to embryo movement. To ask if known components of the Hippo
pathway are expressed in the developing mouse forelimb skeleton, analysis of RNAseq
transcriptome data from Embryonic day (E) 14.5/ Theiler Stage (TS) 23 humeri (ArrayExpress
E-MTAB-1745) was performed. The list of pathway components examined was compiled from
relevant literature and is laid out in Table 1. This revealed that all major components of the
Hippo pathway, including the potential receptor Fat4, core components of the kinase cascade,
the YAP/TAZ effectors, TEAD transcription factors, and known target genes are expressed at
high levels in developing humeri (Fig. 1A, elaborated in Table 1). Further, four of these genes
were more than 2-fold significantly up- or down-regulated when RNA extracted from wildtype
and $Pax3^{Spd/Spd}$ muscle-less mutant humeri were compared (Table 1; Array Express E-MTAB-
1746).

Fat4 and Dchs1 ligand/receptor cell-membrane components were both highly expressed
(>4,000 reads) in wildtype rudiments; additionally, Fat4 was 2.96-fold upregulated in the mutant
(Fig. 1A, Table 1). Other intra-cellular components, acting upstream of YAP/TAZ in the
canonical Hippo pathway (here, collectively referred to as upstream components), were more
moderately expressed, ranging from approx. 200-3,000 reads. The core components (effector
molecules) Yap1 and Taz were highly expressed (>4,000 reads) in both wildtype and mutant.
Among the transcription factors, Tead1 and Tead2 were most highly expressed (approx. 3,000
reads).

Examination of target genes revealed that Ankrd1 had relatively low expression in both the
wildtype and mutant (233 and 2 read counts, respectively) (Fig. 1A, Table 1) and in situ
hybridization showed expression largely restricted to adjacent muscle in the wildtype (data not
shown). The other five target genes examined had high read counts in both wild-type and mutant
(approx. 1,000-7,000) (Fig. 1A, Table 1).

Hippo component genes analyzed here are relatively abundantly expressed in wildtype humeri
and associated joints with 16/25 genes (64.0%) having read counts above 1,000. By
comparison, examining all genes represented in the transcriptome, 33.1% have read counts
over 1,000. (Fig. 1B).
YAP protein shows localized accumulation within the developing skeletal rudiment: levels are elevated at the hypertrophic zone and in regions undergoing morphological expansion. To examine broad patterns of YAP protein localization in the developing limb skeleton, during the period of early sensitivity to stimuli generated by limb movement, we examined the mouse forelimb, focusing on the humerus and associated joints. We detected YAP protein by means of a specific antibody raised against an epitope which spans the primary phosphorylation site, combined with alkaline phosphatase colorimetric staining across Theiler stages 22-24 (E13.5-E15.5), with particular emphasis on TS23 when immobilization effects are first seen most clearly (Fig. 2A). Across stages the strongest levels of detection were in the hypertrophic zones of the humerus and deltoid tuberosity (Fig. 2A a-b, black arrows, arrowhead respectively). With extended staining time, localized patterns of YAP were also detected within the olecranon process of the ulna at the elbow joint (Fig. 2A c-e, arrows) and the coracoid process of the scapula (Fig. 2A j, j'; arrow), becoming more pronounced at TS23 than TS22. Examination of a full set of serial sections through the elbow joint revealed that the most elevated regions of YAP staining are localized within the lateral olecranon process (Fig. 2A d; arrows); while the medial olecranon process also displayed regions of more moderately elevated YAP staining (Fig. 2A e; arrows).

While YAP accumulation is clearly detected in the chondrogenous layers at the joint and in the interzone (Fig. 2A d and j; black arrows), there was reduced staining in a subchondral zone, several cell layers wide (Fig. 2A h', j'; square bracket). These patterns of localized YAP staining in emerging processes and joint regions were not seen with an antibody specific for the phosphorylated version (inactive form) of YAP (pYAP) (Fig. 2A f, f', l, l').

Spatial localization of YAP is altered in the muscle-less mutant. To assess pattern changes in YAP protein under reduced mechanical stimulation, YAP antibody staining was assessed in the Pax3<sup>Spd/Spd</sup> muscle-less mutant forelimb across stages TS22-24, with particular emphasis on TS23 (E14.5) when the phenotypic effects of immobility, including the absence of chondrogenous layers and interzone at presumptive shoulder and elbow joints, are first clearly evident (Nowlan et al., 2010), and were compared to wildtype (Fig. 2). In the muscle-less mutant across stages, YAP protein was not spatially restricted within the processes of the elbow and scapula (olecranon and coracoid), as was evident in the wildtype but was detected at high levels throughout the rudiments (Fig. 2A g, g', k, k', k''; compare to c, c', and j, j', j'' respectively). Additionally, the region of reduced YAP in the elbow and shoulder joint sub-chondral zone of wildtype specimens (Fig. 2A h', j'; brackets) was absent with high levels of detectable YAP across the joint line in muscle-less specimens (Fig. 2A i', k'; black arrow and brackets). There was no difference in the staining pattern of pYAP comparing wildtype and muscle-less humeri (data not shown).
The clear differences in localized accumulation of YAP comparing wildtype and mutant humeri suggested a possible increase in YAP levels overall in the mutant. This was investigated by western blot of protein extracted from microdissected humeri of wildtype and mutant littermates (n=4 litters; 1-3 specimens per phenotype per litter). No significant difference in overall levels of YAP protein was detected between wildtype and mutant (average = 1.18 times YAP protein in mutant compared to wildtype littermates; p=0.56), confirming that spatial localization of YAP protein is affected rather than overall difference in abundance. This difference in spatial localization within the skeletal rudiment is more clearly demonstrated through image analysis of sections from stage-matched mutant and control littermates examined in the same experiment with the same staining conditions. All images were converted to grey-scale and a suitable threshold was applied (identical thresholds were applied to images for comparison). Figure 2A j'' and k'' show the same sections as j' and k', converted to greyscale and thresholded at a level of 120; the localization in the coracoid process evident in Figure 2A j'' was never seen on corresponding mutant sections, and in any of serial sections across the elbow or shoulder joint at stages TS22, 23 or 24.

Since nuclear localization is a hallmark of active YAP, the sub-cellular localization of YAP protein was assessed using immunofluorescent detection. Nuclear localization was clearly distinct (compared to DAPI staining) in the distal head of wildtype humeri (Fig. 2B m, m'; white arrow), although some YAP protein was also detected outside the nucleus (Fig. 2B, m'; yellow arrow). Nuclear YAP was also detected in the cells of the hypertrophic zone (not shown). In the Pax3<sup>Spd/Spd</sup> mutant, as in the wildtype, YAP protein was localized to the nucleus in the distal humeral head (Fig. 2C n'; white arrow), while clear cytoplasmic YAP was also observed in some cells (Fig. 2C n'; yellow arrows).

**YAP target genes Ctgf and Cyr61 show restricted expression in the developing skeleton, and this expression is altered in the muscle-less mutant.** Five YAP target genes commonly used as indicators of YAP transcriptional activity, Ankrd1, Birc5, Ctgf, Col8al, and Cyr61 (Table 1) were selected for expression analysis in wildtype and Pax3<sup>Spd/Spd</sup> muscle-less forelimb rudiments.

**In situ** hybridization of Ctgf and Cyr61 revealed distinct, localized expression patterns within the skeletal rudiments, which were altered in the mutant. In the wildtype, Ctgf expression was detected in the perichondrium and joint chondrogenous layers, lightly outlining the rudiments, and at a low level in the hypertrophic zone (Fig. 3 a-d, red arrows). In the mutant, expression in the perichondrium is increased (Fig. 3 a'-d'), as is expression within the hypertrophic zone (Fig. 3 a'; black arrowhead). In particular, the Ctgf-expressing territory in the region of the chondrogenous layers at the shoulder and elbow joints is strongly increased and expanded in...
the mutant (Fig. 3 b', c'; black arrows). Another striking expression change in the mutant is at
the edge of the olecranon process of the ulna, where staining at the perichondrium was again
expanded and much more intense than in the wildtype (Fig. 3 c'; black arrowhead).

Cyr61 is similarly detected in the chondrogenous layer at the joint and in some regions of the
perichondrium, such as at the hypertrophic zone and at the edge of the olecranon process of
the ulna (Fig. 3 e-g; arrows), but at lower levels than Ctgf. Unlike Ctgf, expression of Cyr61 is
not upregulated in the region of the chondrogenous layers in the mutant (Fig. 3 f'; black arrow);
however elevated expression was observed at a distance, in the subchondral cartilage (Fig. 3
f'; black arrowhead). Expression is also dramatically upregulated in the olecranon process,
similar to Ctgf, but unlike Ctgf it is also detected throughout the olecranon process in the mutant
(Fig. 3 g'; black arrow). In addition, Cyr61 was observed in the prehypertrophic chondrocytes of
the wildtype humerus (Fig. 3 e; red arrowhead), and in the chondrocytes at the mutant
hypertrophic zone (Fig. 3 e'; black arrows), where chondrocyte maturation is delayed compared
to the wildtype (Nowlan et al., 2010).

Birc5 and Col8aI transcripts were detected by RNA-Seq at high levels (Table 4.1; 1,775 and
1,879 read counts, respectively, in the wildtype). Birc5 showed high level expression throughout
the skeletal rudiment with no localized pattern except for more intense staining in the
chondrogenous layers at the joint (Fig. 3 h, i; black arrows). There was no discernible change
in expression in the mutant (Fig. 3 h', i'). Col8aI similarly was detected throughout the skeletal
rudiments with no discernible pattern except for lighter staining at the joint territory in both
wildtype and mutant rudiments (Fig. 3 j-k'; red arrows). It was also detected in the muscles of
the wildtype forelimb (Fig. 3 j, black arrowhead).

Fat4 is not implicated as an upstream regulator of localized YAP activity in developing
skeletal rudiments. To assess a potential role for Fat4, a cadherin receptor and the orthologue
of the Drosophila Hippo pathway receptor Fat (Rock et al., 2005), in regulation of YAP activity
and YAP target gene expression, we performed in situ hybridization for Fat4 on wildtype and
muscle-less mutant tissue. This revealed expression of Fat4 in ectodermal or sub-ectodermal
tissue (Fig. 4 a, c; black arrows), but no Fat4 expression was detected within the rudiments,
which is especially obvious at the joint line (Fig. 4 b, d; red arrows).

We further examined Fat4 null mutants and wildtype littermates to assess if loss of Fat4 might
phenocopy the effects of reduced or absent movement seen in immobilization models such as
Pax3SpdSpd, in particular the typical effects at the joint (loss of chondrogenous layers, joint fusion
and misshapen rudiment termini). In situ hybridization for collagen type 2aI (Col2a1), as a
marker of skeletal rudiment formation, revealed that Fat4 null embryos at E14.5 do not show
the typical immobilization effects at the joint seen in Pax3SpdSpd embryos (compare Fig. 4e and
Fig. 4g shows clear chondrogenous layers (black brackets) and demarcation of the joint line (red arrow) in Fat4<sup>−/−</sup>, unlike the lack of chondrogenous layers, reduced joint line and partial joint fusion seen in Pax<sup>3<sub>Spd</sub>/Spd</sup> (Fig. 4f compared to e, black arrow).

**Inhibition of YAP causes variable effects on chondrogenesis dependent on the timing and level of YAP inactivation.** To manipulate the activity of YAP during chondrogenesis, we used two methods in primary cells in culture: the small molecule verteporfin, which has been shown to inhibit YAP binding to TEAD transcription factors in mammalian and Drosophila cells (Liu-Chittenden et al., 2012), and shRNA knockdown of YAP RNA. Primary cells from the E11.5 limb bud, grown under micromass conditions, spontaneously form a regular pattern of cartilage nodules (Saha et al., 2016) (Fig. 5a) however treatment of cells with 200 nM verteporfin resulted in reduced chondrogenic nodule formation compared to DMSO-treated controls, although nodules still formed in the verteporfin-treated micromasses (Fig. 5b). Expression of YAP target genes *Ctgf* and *Cyr61* were both downregulated in verteporfin-treated cultures, indicative of reduced YAP activity; Cyr61 significantly so, while the reduction in mean *Ctgf* levels was just outside the significance threshold at p=0.058 (Fig. 5c). Overall, the number of nodules formed in verteporfin-treated micromass cultures was significantly reduced, while the size of individual nodules was unchanged (Fig. 5d and data not shown). Inhibition of chondrogenesis by verteporfin treatment in this assay is also shown by reduced chondrogenic marker gene expression: expression of early chondrogenic markers (*Sox9*, *Col2a1*) was significantly downregulated compared to DMSO controls (Fig. 5e). Surprisingly, while expression of hypertrophy marker *Col10a1* was also significantly downregulated after addition of verteporfin, an earlier hypertrophy marker, *Ihh*, was not (Fig. 5e), but this may be related to the assay time point (6 days of culture) when Ihh expression has normally peaked and returned to low levels (Saha et al., 2016).

Contrastingly, inhibition of YAP activity by electroporation of YAP targeted shRNA increased overall chondrogenesis in micromass culture (Fig. 5f-j). This approach might potentially have a delayed effect due to the time required for the electroporated plasmids to undergo transcription and silence RNA transcripts to the extent that overall protein levels are affected. At day 2 of culture, we confirmed reduced YAP protein in electroporated cells by immunostaining for YAP and GFP; electroporated cells as indicated by GFP staining had greatly reduced levels of detectable YAP protein compared to non-electroporated (non-GFP expressing) neighbor cells (Fig. 5f-f'; compare cells indicated by white and yellow arrows). At day 4 of culture, we observed a significant increase in chondrogenic nodules in micromass cultures electroporated with plasmids encoding YAP shRNA, compared to scrambled (NEG) shRNA controls (p<0.05) (Fig. 5g, h), although nodule size remained similar compared to control cultures (data not shown). Note however that the shRNA electroporation approach negatively impacts nodule formation in...
this assay with decreased efficiency of nodule formation in primary cells electroporated with scrambled sequences. It is therefore important to compare each manipulation with its matched negative control (i.e. compare Figure 5 g with h). We conducted four independent experiments, with three experiments showing an approx. 55% reduction in overall YAP mRNA, as detected by qPCR (Fig. 5 j). This corresponded to modest increases in mean levels of chondrogenic marker gene expression (Sox9, Col2a1, Ihh, Col10a1) (1-3 fold), which were not statistically significant (Fig. 5 j), in line with slightly increased nodule formation. A fourth experiment showed a more extreme reduction in levels of YAP mRNA (0.12 compared to control cultures), and consequently showed a drastic reduction in the level of chondrogenic marker gene expression (ranging from 0.003 times the level of Col2a1 to 0.08 times the level of Sox9 in corresponding control micromasses) (data not shown). This suggests that YAP may have an expression threshold at a crucial time in the chondrogenesis process below which differentiation is reduced.
DISCUSSION

A body of previous work has shown the importance of mechanical stimulation generated by embryo movement in the production of a healthy, functioning skeleton; in particular, we have shown the effects of immobilization on appropriate tissue patterning at the joint, normal ossification and shape of skeletal rudiments (reviewed in Shea et al., 2015). However, we have also previously highlighted that there is a major gap in our understanding of how biophysical stimuli generated by movement are transduced to cellular responses in this context. Given the importance of Hippo/Yap signaling in interpreting the mechanical environment, we set out to investigate if YAP might be a mediator of the mechanical signals from movement that guide skeletal development. Two main findings support the proposal:

1) Pathway components are highly expressed in the humerus during the period of early sensitivity to movement and in particular YAP protein shows localized accumulation in key areas when and where tissue shape is changes in the absence of movement; in contrast there is uniform distribution of the inactive form of YAP; pYAP. Further supporting a role for YAP in skeletogenesis, and in line with previous findings (Karystinou et al., 2015; Deng et al., 2016), we demonstrated that blocking YAP pathway activity in micromass culture using an inhibitor reduced chondrogenesis, while knockdown of YAP RNA had an opposing influence indicating that the timing and/or level of YAP is crucial to the effect.

2) In immobile embryos localization of YAP protein and activity of target genes is altered: While overall YAP protein levels are not increased, there is more uniform distribution throughout the rudiment in muscle-less mutant embryos. In particular, localized accumulation in the olecranon and coronoid process, as well as domains of reduced YAP activity in the sub-chondral region distal and proximal to the elbow and shoulder joints in normal embryos are not evident under reduced mechanical stimulation in immobile specimens. Correspondingly there was expanded and increased expression of YAP pathway target genes, indicating increased pathway output in specific territories.

Taken together this provides evidence that YAP signalling is involved in regulating aspects of skeletogenesis, and in particular in mediating the response to mechanical stimulation.

Although YAP was a particularly interesting candidate mechanosensor, very little was known about the activity of the pathway in embryonic skeletal tissue. Here, analysing RNASeq data from the humerus and associated joints at an early stage of mechanosensitivity (E14.5 /TS23) (Nowlan et al., 2010; Rolfe et al., 2014b), we show that all major components of the Hippo-YAP pathway (receptor/ligands; pathway modulators; the main effectors YAP and TAZ; and YAP target genes) are expressed at relatively high levels (Fig. 1). As noted above, the key intracellular mediator, YAP itself, accumulates in specific localized regions of the emerging
tissues, not seen for inactive pYAP across stages TS22-TS24. The highest levels were observed in the hypertrophic zone, with lower levels in the perichondrium, the chondrogenous layers and interzone at the joint, with a region of reduced staining proximal and distal to the joints and localized elevated staining in protrusions of the olecranon and coracoid processes (Fig. 2). Two previous studies examined YAP localization in developing rudiments at different stages and in different rudiments. These studies appeared to be somewhat contradictory where Karystinou et al. (2015) described high levels of nuclear YAP in the perichondrium of E13.5 phalanges and in pre-hypertrophic and hypertrophic chondrocytes at E16.5, while Deng et al. (2016) reported higher levels of nuclear YAP in resting and proliferative chondrocytes compared to hypertrophic chondrocytes in E14.5 tibiae. In E14.5 humeri we see aspects in common with both studies: in hypertrophic zones, perichondrium and some proliferating chondrocyte territories at lower levels, most easily detectable in enlarging protrusions adjacent to the elbow and shoulder joint. Differences might be reconciled by differences in developmental time and specific rudiment since skeletogenesis is a very dynamic process.

Important added value in the current study is the focus on the humerus at a stage demonstrated to be highly mechanosensitive.

The finding of distinct localized patterns of YAP protein within morphological prominences such as the olecranon process of the ulna and the coronoid process of the scapula is particularly novel and important. Such prominences at rudiment termini allow for functional joint articulation and muscle attachment, they undergo enlargement and morphogenesis at this stage and are strikingly sensitive to movement, showing altered shape and growth patterns in immobilization models (Germiller and Goldstein, 1997; Roddy et al., 2011; Nowlan et al., 2014). In particular we have shown correspondence between predicted biophysical stimuli, growth rates and shape changes under immobilization in condyles at the chick knee joint (Roddy et al., 2011). Another striking aspect of the pattern is territories of reduced YAP in the sub-chondral zone, proximal and distal to the shoulder and elbow joint. We have previously shown that territories of Wnt and BMP signaling are distinct and complimentary at rudiment termini with Wnt active in the joint and BMP in the sub-chondral region and that this spatial organization of distinct signaling territories is lost under immobilization in both chick and mouse models (Rolfe et al., 2018; Singh et al., 2018). Here we show that distinct territories of YAP accumulation are also homogenized in the absence of movement.

Examining the effect of immobilization on the YAP pathway is a key part of the study. The transcriptomics data showed that the expression of the majority of components is not altered in immobile embryos. Tead4 and Ankrd1 appear to be down-regulated from a low level of expression in the normal embryo (Table 1), however genes expressed in adjacent muscle in the
wildtype could show low level read counts using this approach due to trace muscle contamination of the sample, which would obviously be entirely absent in the muscle-less mutant. Indeed, *Ankrd1*, a major component of cardiac muscle, was shown here to be highly expressed in adjacent muscle and not detectable in skeletal rudiments by *in situ* hybridization. *Fat4* and *Col8a1* were both significantly upregulated; however, neither showed localized increases in expression by *in situ* hybridization. This could be explained by more widespread upregulation of these genes across tissues, which would not be observable by *in situ* analysis.

The most striking differences observed in muscle-less embryos were at the level of tissue localization of YAP protein itself and two YAP target genes, *Ctgf* and *Cyr61*. YAP protein is more evenly distributed through the rudiment with no distinct localization in morphological features across stages TS22-24 suggesting more widespread signaling activity under reduced mechanical stimulation (Fig. 2). This corresponds to regions of shape change in the muscle-less mutant and in other immobilization models (different genetic strains leading to absent, reduced or immobile muscle and pharmacological immobilization (reviewed in Shea et al., 2015; Rolfe et al., 2018). The shape changes and altered joint formation observed in the immobilization models, including *Pax3*~Spd/Spd~ analyzed here, are not indicative of delayed development with alterations persistent up to at least E18.5 (Kahn et al., 2009) but to ensure that the effect on YAP localization is not a delay, expression was compared in morphologically stage-matched embryos across stages TS22-TS24. The pattern of localized accumulation in the wildtype, demonstrated at TS23 (Fig. 2), is already evident, although more subtle, at TS22 and continues at TS24 (not shown). The *Pax3*~Spd/Spd~ mutant limb showed no localization pattern at TS22, 23 and 24. Conditional overexpression of YAP under the Col2a1 promoter/enhancer was reported to show reduced hypertrophy and mineralization, smaller growth plates, and overall shorter bones (Deng et al., 2016), similar to the phenotypic changes in reduced mechanical stimulation (Kahn et al., 2009; Nowlan et al., 2010). Also in line with our findings, epiphyseal subchondral bone was reduced in YAP-overexpressing mice (Deng et al., 2016), which would affect joint morphology and functionality, as seen under immobilization. To further investigate the effects of immobilization, a panel of known YAP target genes was assessed by *in situ* hybridization. *Ctgf* and *Cyr61* both showed increased and expanded expression in localized regions of rudiments and joints, both genes in hypertrophic zones, and *Ctgf* also in chondrogenous layers and perichondrium, particularly in the olecranon while *Cyr61* increased deeper into the olecranon (Fig. 3). It is notable that both show increased expression in the olecranon where Yap protein is more widespread. These genes encode structurally similar, extracellular matrix proteins belonging to the CCN (*Cyr61/Ccn1, Ctgf/Ccn2, and Nephroblastoma overexpressed/Ccn3*) protein family (reviewed in Krupska et al., 2015). *Ctgf* is expressed in the perichondrium and prehypertrophic/hypertrophic chondrocytes and *Ctgf*-null
mice have expanded hypertrophic domains indicating that it inhibits chondrogenic
maturation/hypertrophy (Friedrichsen et al., 2003; Ivkovic et al., 2003). The increased
expression seen at the mid diaphysis of muscle-less embryos could relate to delayed
hypertrophic progression in muscle-less embryos.

Cyr61 is also well-expressed during skeletal development where it contributes to chondrogenic
differentiation (O'Brien and Lau, 1992; Wong et al., 1997), but in contrast to Ctgf, it has been
suggested to increase chondrocyte hypertrophy (Zhang et al., 2016). The reduction of
expression in muscle-less rudiments at the hypertrophic zone observed here fits with this role.
Cyr61 expression was increased in subchondral cartilage, a territory where morphogenesis is
affected in muscle-less mutants. It is further compelling that in vitro, mechanical stress has
been demonstrated to regulate the expression of both Ctgf and Cyr61 in chondrocytes and other
cell types (reviewed in Chaqour and Goppelt-Struebe, 2006).

Pax3\textsuperscript{Spd/Spd} muscle-less limbs show a very specific set of effects on skeletogenesis that first
becomes evident within a day of the onset of normal embryo movement (Kahn et al., 2009;
reviewed in Shea et al., 2015). There is no observed effect on early chondrogenesis and the
appearance of the cartilage template for each of the rudiments; the effects are seen during the
onset of hypertrophy and most strikingly, the shape of rudiment termini and definition of tissue
territories at the joint, lack of chondrogenous layers and joint fusion, common to multiple
immobilization models. To demonstrate that chondrogenesis can proceed normally in
Pax3\textsuperscript{Spd/Spd} cells we used the micromass culture assay (Saha et al., 2016), preparing cultures
from mutant and wildtype littermate E11.5 limb buds (n=7 and 17 respectively). As expected,
embryonic limb bud cells cultured in high density micromass robustly undergo chondrogenic
differentiation, forming chondrogenic nodules with no difference in the number, size and
timecourse of cartilage nodule formation in cultures prepared from Pax3\textsuperscript{Spd/Spd} limb buds.

To further define the role of YAP during chondrogenesis, micromass cultures were treated with
the YAP-inhibitory molecule verteporfin and YAP RNA was knocked down using shRNA
electroporation. Inhibition of YAP by verteporfin reduced chondrogenesis, as indicated by
Alcian Blue staining and expression of marker genes of chondrogenesis and hypertrophy (Fig.
5). These results indicate that YAP has an early pro-chondrogenic role in this system. This
agrees with other in vitro observations that stiffer substrates, which promote chondrogenesis
and osteogenesis, induce higher levels of nuclear YAP in MSCs (Halder et al., 2012).
Interestingly, in the specific experiments performed here, treatment with shRNA led to more
nodule formation, although no significant effect on chondrogenic marker gene expression. This
could be explained by the delayed effects of RNA silencing, compared to the immediate effect
of drug treatment and/or a milder disturbance of YAP protein. Early inhibition of YAP may delay
the earliest stages of chondrogenic nodule formation, while later inhibition may not have the
same effect and even permit the formation of more foci of cellular condensation; more nodules.
Indeed, Deng et al. (2016) and Karystinou et al. (2015), both demonstrate that YAP protein
levels decrease over time and YAP may have different roles as chondrogenesis progresses. It
is also important to note that the shRNA approach causes a reduction in chondrogenesis in this
assay system, which may affect the sensitivity of the assay, although all comparisons are made
with the appropriate control.

While regulation of Hippo pathway activity at the receptor level is well-defined in Drosophila, the
mechanism of upstream control is unclear in the mammalian system. We therefore examined
Fat4 as a potential regulator of Hippo signaling in this context, as it is the mammalian orthologue
of the cell-surface receptor in Drosophila, Fat. The role of Fat4 in mammalian Hippo signaling
is not well-defined, and other mechanoresponsive mechanisms which can regulate YAP
transcriptional activity, such as cytoskeletal tension, have been identified (Dupont et al., 2011).
The effects of the Fat4 mutation have previously been analyzed in a number of organ systems
including the skeleton where Mao et al. (2016) demonstrated a role for Fat4 in planar cell
polarity, specifically during elongation of the sternum, but independent of YAP. Recently,
Crespo-Enriquez, et al. (2019) showed that Fat4 is essential for osteoblast differentiation and
that mutants have abnormalities in the cranial skeleton, but the only difference seen in the
appendicular skeleton at P0 was a decrease in cortical thickness at the midshaft. The specific
objective of the analysis presented in this study was to ask if Fat4−/− embryos phenocopy the
effects in immobile embryos, specifically the effects at the joint where we see loss of
chondrogenous layers, joint fusions and misshapen condyles and if Fat4 is expressed in the
affected territories. Fat4 expression was detected in the developing limb from E10.5-E14.5, but
this did not correspond with mesenchymal condensations or later skeletal rudiments (Fig. 4 and
unpublished data). Although we did observe expression of Fat4 across the territory of the
developing knee and elbow joints between E11.5 and E12.5 and the digit joints at E14.5 (data
not shown), Fat4-null mice showed no obvious defects in joint formation, suggesting that Fat4
is not required for normal joint patterning nor cavitation. While Fig. 4f shows the typical
phenotype of Pax3<sup>spd/spd</sup> (and Myf5<sup>−/−</sup>/MyoD<sup>−/−</sup> and immobile muscle) with reduced joint line,
absence of chondrogenous layers and partial fusion of rudiments, the Fat4<sup>−/−</sup> mutant elbow joint
(Fig. 4g) shows clear chondrogeous layers (typified by increased cell density and orientation of
cells along the joint line) and separation of the rudiments. This opens the possibility of non-
canonical mechanisms of YAP activation in this context, such as binding to Angiomotin (Zhao
et al., 2011; Leung and Zernicka-Goetz, 2013), or changing the cytoskeleton and its associated
proteins. Numerous cytoskeletal components were found to be differentially regulated in the
muscle-less mouse humerus and associated joints (Rolfe et al., 2014b), and so YAP activity
may be altered by cytoskeletal changes under reduced mechanical stimulation.

To refine the role of YAP and its upstream regulation during skeletal development, conditional
mutants or other in vivo functional studies are required. YAP conditional ectopic expression
under the Col2a1 promoter/enhancer resulted in smaller skeletal rudiments when YAP is
overexpressed during later stages of chondrogenesis (Deng et al., 2016). Ctgf and Cyr61 target
gene expression was shown to increase by qRT-PCR, but it would be interesting to investigate
any spatial changes in the expression. While conditional expression or deletion under Col2a1
control is a valuable tool to examine later cartilage maturation and hypertrophy, conditional over
or under expression at earlier time points, under the control of earlier markers of skeletal
development, such as the Sox9 transcription factor, would allow for assessment of YAP’s role
in early skeletogenesis. Likewise, restriction of YAP deletion to the joint region, for example
under the control of Gdf5, could be used to explore the role of YAP in the differentiation of
articular cartilage.

This study implicates YAP signaling in the mechanoresponsiveness of skeletal tissue
differentiation during embryonic development for the first time. While skeletal development has
previously been demonstrated to be acutely mechanosensitive, a mechanism for the capacity
of cells to sense and respond to mechanical stimuli has been missing. Here, we propose that
YAP serves as such a mechanoresponder, capable of coordinating a molecular response to
mechanical stimuli. Furthermore, the observed alteration in active YAP distribution under
reduced mechanical stimulation may contribute to the effect on skeletogenesis, in particular
patterns of tissue differentiation at the joint and emergence of shape, known to require
appropriate movement during early development.

EXPERIMENTAL PROCEDURES

Animal lines. Outbred CD-1 and heterozygous Splotch delayed (Pax3Spd+/) (Vogan et al., 1993)
(acquired from Jackson Laboratories) mouse lines were bred and euthanized under the
supervision and approval of the Trinity College Bioresources Unit and the Bioethics committee
and under personal licenses from the Health Products Regulatory Authority. Splotch delayed
mutant embryos were generated by crossing heterozygous males and females. Embryos were
harvested at embryonic days (E) 10.5-14.5 of gestation, precisely staged based on
morphological criteria (Theiler Stage (Theiler, 1989) (all comparisons made are between
morphologically stage-matched embryos), fixed in 4% paraformaldehyde overnight, dehydrated
to absolute methanol and stored at -20°C for later tissue analysis. Genotype was confirmed by
PCR amplification of the mutant or wildtype allele (Keller-Peck and Mullen, 1997). Transcriptomic
analysis of micro-dissected humeri was previously performed (Rolfe et al.,
Differential expression was defined as more than 2-fold up- or down-regulated, with an adjusted p-value of ≤ 0.05. Adjusted p-values were calculated according to the Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995), which controls for the false discovery rate (FDR) (i.e. reduces false positives). *Fat4* null embryos were received from the McNeill lab (Saburi et al., 2008).

**Immunohistochemistry.** Limbs were rehydrated from storage in methanol and either cryoprotected in 30% sucrose in PBS and frozen for cryosectioning, or paraffin embedded and sectioned. Paraffin sections were dewaxed with Histo-Clear II (National Diagnostics) and rehydrated before immunolabelling. Heat-mediated antigen retrieval was performed on sections (10μm) for YAP immunolabelling by immersing slides in boiling 0.01 M sodium citrate buffer (pH 6) and incubating at 90°C for 20 minutes. Sections were blocked in 5% goat serum in TBS with 0.1% Tween (TBST). Primary antibody was applied overnight, at 4°C. Primary antibodies used were: mouse monoclonal anti-YAP (63.7) (Santa Cruz cat. no.: sc-101199, diluted 1:100 in blocking buffer) and rabbit polyclonal anti-phospho-YAP, Ser127 (Cell Signalling, cat. no.: 4911, 1:200). For colorimetric detection, goat anti-mouse or anti-rabbit IgG-Alkaline Phosphatase secondary antibodies were used; sections were equilibrated to pH 9 in NMT buffer (0.1 M Tris pH 9.5, 0.05 M MgCl₂, 0.1% Tween), then developed with NBT/BCIP diluted in NMT. Short staining periods revealed YAP localization in the hypertrophic zones at the mid diaphysis and deltoid tuberosity whereas extended staining showed the consistent patterns described at the joint and within expanding condyles (all slides compared (all genotypes and antibodies (Yap, pYAP and negative control) were stained under the same conditions for the same period). Controlled image analysis was used to compare the localization of YAP staining observed in wildtype and *Pax3<sup>Spd/Spd</sup>* skeletal rudiment termini. Serial sections processed and stained identically were converted to grey-scale and threshold values from 90 to 190 (increments of 10) applied (ImageJ). For fluorescent detection of YAP, a biotinylated goat anti-mouse secondary antibody (Santa Cruz cat. no.: sc-2039, 1:250) was applied, followed by streptavidin-HRP (1:400) and a tyramide substrate labelled with AlexaFluor 568 (Invitrogen T20934), according to manufacturer’s instructions. Fluorescent samples were mounted with ProLong Gold Antifade Reagent with DAPI (Life Technologies) or stained with DAPI (Sigma) and mounted in Mowiol with 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma).

**Protein Extraction and Western Blot.** *Pax3<sup>Spd</sup>* embryos were harvested at E14.5 and dissected. Embryos were assessed phenotypically as wildtype or mutant, and genotyped, as described above. Limbs were sub-dissected and humeri were placed in cold PBS supplemented with a mini cOmplete protease inhibitor (Roche), 1mM EDTA, 10 mM sodium fluoride, and 25 mM sodium pyrophosphate to prevent protein degradation. Following subdissection, humeri were stored in TRIzol (Thermo Fisher Scientific) at -80°C. The
manufacturer’s protocol for protein extraction was followed and extracted total protein was resuspended in 0.1% SDS. Individual specimens were pooled by genotype, and total protein quantified (Qubit 2.0 fluorometer, Invitrogen) and stored at -20°C. For Western blotting, 5-10μg of protein was separated on a 10% Acryl-Bis gel. PVDF membranes (0.45μm, Amersham) were blocked with 5% milk in TBST and incubated in 1:1000 anti-YAP or 1:3000 anti-HDJ2 (Thermo Fisher Scientific), followed by 1:2000 anti-mouse-HRP (Thermo Fisher 626520). Densitometry analysis was performed using Adobe Photoshop, comparing YAP protein totals between wildtype and mutant littermate samples (n=4 independent litters); YAP protein levels were normalized to HDJ2 protein levels prior to wildtype/mutant comparison.

**In situ hybridization.** Antisense RNA probes were generated from specific cDNA clones (Table 2). RNA was produced by *in vitro* transcription using T7, T3, or SP6 RNA polymerases and DIG-labelled nucleotides (Roche) and probes were purified with Illustra MicroSpin G-25 columns (GE) and quantified with a Qubit 2.0 Fluorometer (Invitrogen). Hybridization of 60μm vibratome sections or 10μm cryosections was carried out largely as described previously (Nowlan et al., 2008; Summerhurst et al., 2008; Rolfe et al., 2014b).

**Micromass culture, verteporfin treatment, and electroporation.** Micromass cultures were prepared as described in Saha et al. (2016) from outbred CD1 E11.5 limb buds and from Pax3Spd/Spd and wildtype littermate embryos (4-6 individual cultures per embryo). Cells were plated in 10-15 μl drops at 1-2 x 10^7 cells/mL either directly on tissue-culture grade treated plastic dishes (Thermo Fisher/Nunc), or on 10-mm glass coverslips treated with 0.015 M poly-D-ornithine (Sigma). After plating, micromass cultures were allowed to adhere for at least one hour in incubation conditions (37°C and 5% CO2), then gently flooded with 400 μl of growth medium (DMEM F12 (D8427), Penicillin-Streptomycin (100U/ml, P4333), L-Glutamine (2mM, G7513), 10% FBS and Ascorbic acid (50ug/ml, A8960) (All Sigma)) per well.

For Verteporfin experiments, experimental cultures were treated for the entire culture period with Verteporfin (200 nM in DMSO, SML0534 Sigma) in growth medium, while control cultures were in growth medium supplemented with an equivalent volume of DMSO. Medium was fully changed on day 2 and day 4, and micromasses were harvested at Day 6 for Alcian Blue staining or qRT-PCR analysis.

For shRNA treatment, shRNA-encoding plasmids were purchased from the Mission shRNA library of bacterial clones (Sigma). As a negative control, non-target shRNA control plasmid DNA (Sigma SHC216) was used to transform chemically competent bacteria, amplified, and purified using a DNA maxiprep kit (Qiagen, UK). Purified plasmids were further concentrated by ethanol-sodium acetate precipitation and centrifugation, before final resuspension in 0.1M Tris-HCl buffer, and quantification with the Qubit system (Thermo Fisher Scientific).
Electroporation of cells was conducted using the Neon Transfection system, with 100 µl volume gold pipette tips (Thermo Fisher Scientific). Limb bud cells were prepared as described above except penicillin-streptomycin was omitted prior to electroporation. After cell counting, cells were spun down and resuspended in Neon transfection system resuspension buffer R, to give a final concentration of 3 x 10^7 cells/mL; assuming 50% cell death, this gives the desired final cell concentration of 1.5 x 10^7 cells/mL. The shRNA plasmid combined with a GFP expression construct (give ration) was added to the cells, at a final concentration of 10 µg DNA/1 x 10^6 cells. The following parameters were used for electroporation, following optimization: 1400 V, 2 pulses with 20 ms interval. Following electroporation, cells were added to a small amount of growth medium and plated as 10 µl spots on plastic or treated coverslips, as described above. Micromasses were allowed to adhere for 2 hours, then flooded with 400 µl of growth medium.

**Micromass Processing.** For Alcian Blue staining, cells were fixed for 30 minutes at room temperature in 4% PFA and stored in PBS at 4°C. Staining was performed in 1% Alcian Blue 8X (Sigma) in HCl for 30 minutes at room temperature and imaged in PBS or mounted with Aqua-Poly/Mount. For qRT-PCR, cells were incubated with 100 µl of TRIzol reagent for 2 minutes; each well was rinsed and scraped several times to fully detach cells. Several wells (2-4 per sample) were pooled to ensure sufficient RNA quantity. cDNA was reverse transcribed and amplified with SYBR green detection, using predesigned Kicqstart primers (Sigma-Aldrich) (Table 3). Plates were run with triplicate samples on an Applied Biosystems 7500 Real-Time PCR system. \( \Delta C_t \) values were calculated by normalising the cycle threshold values for each gene to the endogenous control gene, GAPDH. Gene expression was then calculated as the fold change relative to a reference sample \( (2^{-\Delta \Delta C_t}) \). Cycle threshold data were compared to data for control samples; for Verteporfin treatment, the reference samples were DMSO-treated; for shRNA treatment the control sample was electroporated with non-target shRNA control plasmid DNA (Sigma SHC216). Statistical significance was determined using Student’s t-tests, with significance defined at a p-value of ≤ 0.05.

**Microscopy and image analysis.** Fluorescent images were collected on an Olympus DP72 camera with CellSens software (v1.6) and a Leica SP8 scanning confocal microscope with Leica Application Suite software (LAS v5.1). Images were analyzed using ImageJ software (Version 1.51j8, National Institutes of Health, Bethesda, MD.). Original TIFF (.tif) files were cropped to a circle of 5 mm diameter and cleaned to remove dust or other debris. Images were converted to an 8-bit black and white image by setting a minimum threshold, using the auto-threshold function as a guideline. Treatment groups within an experiment were thresholded at the same value, but the appropriate threshold values were determined independently by eye for each experiment, due to unavoidable variability in staining...
640 contrast or intensity, and in image capture. Noise was removed and converged nodules were
641 separated using the Despeckle and Watershed functions, respectively. Nodules were counted
642 and measured using the Analyze Particles function in ImageJ, with a minimum particle size of
643 0.005 µm². Nodules touching the edge of the circular frame were excluded to prevent inaccurate
644 size measurements (as these nodules are partially outside the frame of measurement).

645 **ACKNOWLEDGEMENTS**
646 This research was funded by an Irish Research Council Postgraduate Studentship (2013/233)
647 and the Wellcome Trust (083539/Z/07/Z). We thank Jamie Kelly and Niamh Rudden for
648 assistance with preliminary immunostaining and *in situ* hybridization.
REFERENCES


Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. Dev Cell 16:398-410.


FIGURE LEGENDS

Figure 1. Multiple components of the Hippo pathway are relatively highly expressed in the E14.5 developing humerus and associated joints. A. A schematic representation of the mammalian Hippo pathway; pathway components are presented as boxes proportional to their RNA read count in wildtype tissue (Table 1). B. The number of genes (y axis) in each transcript abundance category (x-axis) for the entire transcriptome (black bars) and for Hippo pathway genes (grey bars).

Figure 2. YAP protein shows localized elevation in specific regions of the developing humerus which is lost in the muscle-less Pax3SpdlSpd mutant. A. Paraffin sections of E14.5(TS23) wildtype or mutant forelimbs stained for YAP protein or phosphorylated YAP (pYAP). (a) Whole humerus (asterisk indicates position of b) on adjacent section; (b) deltoid tuberosity (arrowhead) with adjacent hypertrophic zone (arrow). (c-g’) sections of the humeral-ulnar elbow joint, showing the olecranon process; (d) and (e) are more lateral and medial sections, respectively, compared to (c’). (h-i’) show the humeral-radial elbow joint. (j-l’) show the shoulder joint. (f-f’, l, l’) show staining for pYAP (outlined in orange boxes). (g, g’, i, i’, k, k’) mutant specimens (outlined in red box). j” and k” are grey-scale thresholded versions of j’ and k’. Black arrows/brackets and red brackets indicate expression or absent expression respectively, as described. Yellow dashed boxes indicated regions of higher magnification in adjacent panels. Abbreviations: humerus (H), radius (R), ulna (U), scapula (S). Scale bars: 100 µm. B. Immunofluorescent detection of YAP (red), counterstained with DAPI. (m, m’) wildtype tissue; (n, n’) muscle-less mutant tissue; all panels show the distal humerus. Yellow and white arrows indicate cytoplasmic and nuclear staining, respectively. Scale bars: 10 µm.

Figure 3. YAP target genes Ctgf and Cyr61 show localized expression in the skeletal rudiments which is altered in the muscle-less (Pax3SpdlSpd) mutant. Expression of YAP target genes Ctgf, Cyr61, Birc5, and Col8a1, comparing wildtype forelimb cryosections (E14.5) (a-k) to muscle-less mutant tissue (Pax3SpdlSpd) (a’-k’). Black and red arrows indicate expression as described in the text. Dashed yellow lines indicate presumptive joint-lines. Regions of expression included the humeral diaphysis midpoint (a, a’, e, e’, j, j’), the shoulder joint (b, b’, f, f’, i, i’, k, k’), the humero-ulnar joint of the elbow (c, c’, g, g’, h, h’), and the humero-radial joint of the elbow (d, d’). Abbreviations: humerus (H), radius (R), ulna (U). Scale bars are 100 µm.

Figure 4. Fat4 is not implicated as an upstream regulator of localized YAP activity in developing skeletal rudiments. (a-d) 60 µm vibratome sections of wildtype (a-b) or muscle-less (c-d) forelimbs at TS23, in situ hybridized for Fat4 expression. Black and red arrows indicate expression or lack of expression as described in the text. (a and c) show the whole humerus; (b and d) show the elbow joint. (e-g) shows sections though the elbow joint of
*Pax3* Spd/*Spd* muscleless mutant and *Fat4* mutant embryos compared to normal littermates at TS23, stained following *in situ* hybridization for Col2a1 expression, as indicated. Note the reduction of the joint line and lack of chondrogenous layers in *Pax3* Spd/*Spd* mutant embryos while chondrogenic rudiments appear normal in *Fat4* null. Square brackets indicate the chondrogenous layers in g. Scale bars are 100 µm.

**Figure 5. Early inhibition of YAP decreases *in vitro* chondrogenesis in micromass culture.** Micromass cultures were treated with DMSO (a) or with 200 nM Verteporfin in DMSO (b) for 6 days, fixed, and stained with Alcian Blue (representative images shown). Gene expression of YAP targets *Ctgf* and *Cyr61* (c) and of chondrogenic markers (e) in micromasse cultures, as detected by qRT-PCR. Images of stained cultures were analyzed for nodule number (d). *n*=2 micromass cultures in each treatment group, for 3 independent experiments in all cases a-e. Constructs encoding shRNA for YAP or a mis-sense control (NEG) were co-electroporated with a GFP-encoding construct into limb bud cells prior to micromass plating (f-j). (f, f') immunostaining for YAP and GFP; white arrow indicates GFP-expressing cells, while yellow arrow indicates GFP-negative cells. Micromass cultures harvested on day 2 of culture were stained with Alcian Blue (g, h) and nodule number quantified (i). Gene expression of YAP and chondrogenic markers were assessed by qRT-PCR (j). *n*=2-4 micromass cultures in each group, for 4 independent experiments. Abbreviations: verteporfin (VP). Scale bars in (a, b, g, h) are 1 mm. Significance: *p*<0.05 (*), *p*<0.005 (***) *p*<0.0001 (****). Error bars represent standard error.
### Table 1. Expression of key Hippo pathway genes in the developing humerus. ArrayExpress reference E-MTAB-1745 and E-MTAB-1746,

<table>
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<tr>
<th>Gene Symbol</th>
<th>Gene name</th>
<th>Role in mammals (Reference)</th>
<th>Drosophila homolog (gene symbol)</th>
<th>Wildtype reads</th>
<th>Mutant reads</th>
<th>Fold Change</th>
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<td>Fat4</td>
<td>FAT atypical cadherin 4</td>
<td>Similar cytoplasmic domain to <em>Drosophila Fat</em> (Tanoue and Takeichi, 2005); involved in PCP pathway (Saburi et al., 2008); role in Hippo unclear</td>
<td>Dachsous (Ds)</td>
<td>4,367</td>
<td>12,916</td>
<td>2.95</td>
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<td>Dchs1</td>
<td>Dachsous cadherin-related 1</td>
<td>Interacts with Fat4 to regulate PCP (Mao et al., 2011; Kuta et al., 2016); no clear role in Hippo</td>
<td>Dachsous (Ds)</td>
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<td>Fjx1</td>
<td>Four jointed box 1</td>
<td>Possibly regulates Fat4/Dchs1 binding (Rock et al., 2005)</td>
<td>Four-jointed</td>
<td>980</td>
<td>689</td>
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<td>Nf2</td>
<td>Neurofibromatosis type 2</td>
<td>Tumor suppressor which complexes with Kibra and Sav1 to inactivate YAP (Zhang et al., 2010)</td>
<td>Merlin</td>
<td>2,814</td>
<td>3,132</td>
<td>1.11</td>
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<td>Wwc1 (Kibra)</td>
<td>WW and C2-domain containing protein 1 (Kibra)</td>
<td>Stimulates phosphorylation of LATS1/2 (Xiao et al., 2011)</td>
<td>Kibra</td>
<td>188</td>
<td>176</td>
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<tr>
<td>Frmd6 (Willin)</td>
<td>FERM-domain containing protein 6</td>
<td>Activates MST1/2 (Angus et al., 2012)</td>
<td>Expanded (Ex)</td>
<td>2,030</td>
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<td>Sav1</td>
<td>Salvador homolog 1</td>
<td>Associates with MST1/2, promotes LATS1/2 phosphorylation (Callus et al., 2006)</td>
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<td>1,898</td>
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<td>Mob1a (Mobkl1a)</td>
<td>MOB kinase activator 1A</td>
<td>Phosphorylation by MST1/2 allows for binding/activation of LATS1/2 (Praskova et al., 2008)</td>
<td>Mob as tumor suppressor (Mats)</td>
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<td>Mst1 (Stk4)</td>
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<td>Phosphorylates LATS1/2, MOB1A/B, and SAV1 (Chan et al., 2005; Callus et al., 2006; Praskova et al., 2008)</td>
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<td>Phosphorylates YAP (Oka et al., 2008)</td>
<td>Warts (Wts)</td>
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<td><strong>Yap1</strong></td>
<td>Yes-Associated Protein 1</td>
<td>Transcriptional co-activator/effect of the Hippo pathway (Yagi et al., 1999; Huang et al., 2005b; Zhang et al., 2010)</td>
<td>4,857</td>
<td>5,121</td>
<td>1.06</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td></td>
<td><strong>Taz</strong> (Wwtr1)</td>
<td>Transcriptional activator with PDZ-binding motif (WW-domain containing transcription regulator 1)</td>
<td>Transcriptional co-activator/effect of the Hippo pathway (Lei et al., 2008)</td>
<td>4,107</td>
<td>4,081</td>
<td>0.99</td>
<td>0.783</td>
</tr>
<tr>
<td></td>
<td><strong>TRANSCRIPTION FACTORS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tead1 (Tef1)</td>
<td>TEA-domain family member 1</td>
<td>(Transcriptional enhancer factor 1)</td>
<td></td>
<td>2,966</td>
<td>2,919</td>
<td>0.99</td>
<td>0.9916</td>
</tr>
<tr>
<td>Tead2 (Tef4)</td>
<td>TEA-domain family member 2</td>
<td>(Transcriptional enhancer factor 4)</td>
<td>TEA-domain transcription factors bound by YAP/TAZ (Zhao et al., 2008b)</td>
<td>3,800</td>
<td>2,819</td>
<td>0.74</td>
<td>0.5507</td>
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<tr>
<td>Tead3 (Tef5)</td>
<td>TEA-domain family member 3</td>
<td>(Transcriptional enhancer factor 5)</td>
<td></td>
<td>919</td>
<td>953</td>
<td>1.04</td>
<td>&gt;0.9999</td>
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<tr>
<td>Tead4 (Tef3)</td>
<td>TEA-domain family member 4</td>
<td>(Transcriptional enhancer factor 3)</td>
<td></td>
<td>265</td>
<td>69</td>
<td>0.26</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vgll4</td>
<td>Vestigial-like family member 4</td>
<td></td>
<td>Transcription factor which competes with TEADs to bind YAP/TAZ and inhibit transcriptional activity (Guo et al., 2013)</td>
<td>531</td>
<td>913</td>
<td>1.72</td>
<td>0.0419</td>
</tr>
<tr>
<td></td>
<td><strong>DOWNSTREAM TRANSCRIPTIONAL TARGETS</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ankrd1</td>
<td>Ankyrin repeat domain-containing protein 1</td>
<td></td>
<td>Skeletal muscle component; target of YAP (Zanconato et al., 2015)</td>
<td>233</td>
<td>2</td>
<td>0.01</td>
<td>0.0001</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Gene name</td>
<td>Role in mammals (Reference)</td>
<td>Drosophila homolog (gene symbol)</td>
<td>Wildtype reads</td>
<td>Mutant reads</td>
<td>Fold Change</td>
<td>Adjusted P-value</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
<td>------------------------------</td>
<td>---------------------------------</td>
<td>----------------</td>
<td>--------------</td>
<td>-------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Birc5</td>
<td>Baculoviral inhibitor of apoptosis repeat-containing 5 (Survivin)</td>
<td>Inhibitor of apoptosis; target of YAP (Dong et al., 2007)</td>
<td></td>
<td>1,775</td>
<td>1,223</td>
<td>0.69</td>
<td>0.6008</td>
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<tr>
<td>Ctgf (Ccn2)</td>
<td>Connective tissue growth factor (CCN family member 2)</td>
<td>Heparin-binding matricellular protein; target of YAP (Zhao et al., 2008b)</td>
<td></td>
<td>2,714</td>
<td>3,633</td>
<td>1.34</td>
<td>0.6374</td>
</tr>
<tr>
<td>Cyr61 (Ccn1)</td>
<td>Cystein-rich angiogenic inducer 61 (CCN family member 1)</td>
<td>Promotes endothelial cell adhesion; target of YAP (Zhang et al., 2008)</td>
<td></td>
<td>2,658</td>
<td>3,146</td>
<td>1.18</td>
<td>0.6374</td>
</tr>
<tr>
<td>Inha</td>
<td>Inhibin alpha</td>
<td>FSH secretion inhibitor; target of YAP (Piccolo et al., 2014)</td>
<td></td>
<td>197</td>
<td>123</td>
<td>0.62</td>
<td>0.4949</td>
</tr>
<tr>
<td>Axl</td>
<td>AXL receptor kinase</td>
<td>Tyrosine kinase; target of YAP (Zanconato et al., 2015)</td>
<td></td>
<td>5,409</td>
<td>6,671</td>
<td>1.23</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Col8a1</td>
<td>Collagen VIII alpha I</td>
<td>Structural component of extra-cellular matrix; target of YAP (Zhang et al., 2008)</td>
<td></td>
<td>1,879</td>
<td>4,312</td>
<td>2.30</td>
<td>0.0001</td>
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</table>
Table 2. Details of antisense RNA probes used.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>GenBank RefSeq</th>
<th>Nucleotides in reference sequence corresponding to probe</th>
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</thead>
<tbody>
<tr>
<td>Ankrd1</td>
<td>NM_013468.3</td>
<td>12-1765</td>
</tr>
<tr>
<td>Birc5</td>
<td>NM_001012273.1</td>
<td>1424-3416</td>
</tr>
<tr>
<td>Col2aI</td>
<td>NM_031163.3</td>
<td>4,678-5,083</td>
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<tr>
<td>Col8aI</td>
<td>NM_007739.2</td>
<td>34-2468</td>
</tr>
<tr>
<td>Ctgf</td>
<td>NM_010217.2</td>
<td>80-2418</td>
</tr>
<tr>
<td>Cyr61</td>
<td>NM_010516.2</td>
<td>15-2028</td>
</tr>
<tr>
<td>Fat4</td>
<td>NM_183221.3</td>
<td>13,940-14,924</td>
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Table 3. Details of primers used in qRT-PCR.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>RefSeq</th>
<th>Primer Sequences</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>NM_008084</td>
<td>Fwd: TGGCCTCCAAGGAGTAAGAAAC&lt;br&gt;Rev: GGGATAGGCTCCTCCTTTGCT</td>
</tr>
<tr>
<td>Ctgf</td>
<td>NM_010217.2</td>
<td>Fwd: GAGGAAAACATTAAGAAGGGC&lt;br&gt;Rev: AGAAAGCTCAAACTTGACAG</td>
</tr>
<tr>
<td>Cyr61</td>
<td>NM_010516.2</td>
<td>Fwd: AGAGGCTTCCTGTCTTTG&lt;br&gt;Rev: GGTTCATTTGTAACTCGTG</td>
</tr>
<tr>
<td>YAP</td>
<td>NM_001171147.1</td>
<td>Fwd: GATGTTCAGGAATGGAGAC&lt;br&gt;Rev: CTGTATCCATTTGACAC</td>
</tr>
<tr>
<td>Col2aI</td>
<td>NM_01163.3</td>
<td>Fwd: AAGTCACTGAACAACCAGATTGAAG&lt;br&gt;Rev: AAGTGCAGGAGGGTCTTTG</td>
</tr>
<tr>
<td>Sox9</td>
<td>NM_011448.4</td>
<td>Fwd: CGGCTCCAGCAAGAACAAG&lt;br&gt;Rev: TGCGGCCACCATCGTA</td>
</tr>
<tr>
<td>Ihh</td>
<td>NM_010544.2</td>
<td>Fwd: CCACCTCAGTGTAGGTCTTTAT&lt;br&gt;Rev: CAGAGGAGTACAGGCCGTTTG</td>
</tr>
<tr>
<td>Col10aI</td>
<td>NM_009925.4</td>
<td>Fwd: TGCAATCATGGAGCCCTCAGA&lt;br&gt;Rev: CAGAGGAGTACAGGCCGTTTG</td>
</tr>
</tbody>
</table>
Figure 1. Multiple components of the Hippo pathway are relatively highly expressed in the E14.5 developing humerus and associated joints.

A. A schematic representation of the mammalian Hippo pathway; pathway components are presented as boxes proportional to their RNA read count in wildtype tissue (Table 1).  B. The number of genes (y axis) in each transcript abundance category (x-axis) for the entire transcriptome (black bars) and for Hippo pathway genes (grey bars).
Figure 2. YAP protein shows localized elevation in specific regions of the developing humerus which is lost in the muscle-less Pax3Spd/Spd mutant.

A. Paraffin sections of E14.5(TS23) wildtype or mutant forelimbs stained for YAP protein or phosphorylated YAP (pYAP). (a) Whole humerus (asterisk indicates position of b) on adjacent section; (b) deltoid tuberosity (arrowhead) with adjacent hypertrophic zone (arrow). (c-g’) sections of the humeral-ulnar elbow joint, showing the olecranon process; (d) and (e) are more lateral and medial sections, respectively, compared to (c’). (h-i’) show the humeral-radial elbow joint. (j-l’) show the shoulder joint. (f-f’, l, l’) show staining for pYAP (outlined in orange boxes). (g, g’, i, i’, k, k’) mutant specimens (outlined in red box). (g, g’, i, i’, k, k’) grey-scale thresholded versions of j’ and k’. Black arrows/brackets and red brackets indicate expression or absent expression respectively, as described. Yellow dashed boxes indicated regions of higher magnification in adjacent panels. Abbreviations: humerus (H), radius (R), ulna (U), scapula (S). Scale bars: 100 µm.

B. Immunofluorescent detection of YAP (red), counterstained with DAPI. (m, m’) wildtype tissue; (n, n’) muscle-less mutant tissue; all panels show the distal humerus. Yellow and white arrows indicate cytoplasmic and nuclear staining, respectively. Scale bars: 10 µm.
Figure 3. YAP target genes Ctgf and Cyr61 show localized expression in the skeletal rudiments which is altered in the muscle-less (Pax3Spd/Spd) mutant. Expression of YAP target genes Ctgf, Cyr61, Birc5, and Col8a1, comparing wildtype forelimb cryosections (E14.5) (a-k) to muscle-less mutant tissue (Pax3Spd/Spd) (a'-k'). Black and red arrows indicate expression as described in the text. Dashed yellow lines indicate presumptive joint lines. Regions of expression included the humeral diaphysis midpoint (a, a', e, e', j, j'), the shoulder joint (b, b', f, f', i, i', k, k'), the humero-ulnar joint of the elbow (c, c', g, g', h, h'), and the humero-radial joint of the elbow (d, d'). Abbreviations: humerus (H), radius (R), ulna (U). Scale bars are 100 µm.
Figure 4. Fat4 is not implicated as an upstream regulator of localized YAP activity in developing skeletal rudiments. (a-d) 60 µm vibratome sections of wildtype (a-b) or muscle-less (c-d) forelimbs at TS23, in situ hybridized for Fat4 expression. Black and red arrows indicate expression or lack of expression as described in the text. (a and c) show the whole humerus; (b and d) show the elbow joint. (e-g) shows sections though the elbow joint of Pax3Spd/Spd muscleless mutant and Fat4 mutant embryos compared to normal littermates at TS23, stained following in situ hybridization for Col2a1 expression, as indicated. Note the reduction of the joint line and lack of chondrogenous layers in Pax3Spd/Spd mutant embryos while chondrogenic rudiments appear normal in Fat4 null. Square brackets indicate the chondrogenous layers in g. Scale bars are 100 µm.
Figure 5. Early inhibition of YAP decreases in vitro chondrogenesis in micromass culture.

Micromass cultures were treated with DMSO (a) or with 200 nM Verteporfin in DMSO (b) for 6 days, fixed, and stained with Alcian Blue (representative images shown). Gene expression of YAP targets Ctgf and Cyr61 (c) and of chondrogenic markers (e) in micromasse cultures, as detected by qRT-PCR. Images of stained cultures were analyzed for nodule number (d). n=2 micromass cultures in each treatment group, for 3 independent experiments in all cases a-e. Constructs encoding shRNA for YAP or a mis-sense control (NEG) were co-electroporated with a GFP-encoding construct into limb bud cells prior to micromass plating (f-j). (f, f') Immunostaining for YAP and GFP; white arrow indicates GFP-expressing cells, while yellow arrow indicates GFP-negative cells. Micromass cultures harvested on day 2 of culture were stained with Alcian Blue (g, h) and nodule number quantified (i). Gene expression of YAP and chondrogenic markers were assessed by qRT-PCR (j). n=2-4 micromass cultures in each group, for 4 independent experiments. Abbreviations: verteporfin (VP). Scale bars in (a, b, g, h) are 1 mm. Significance: p<0.05 (*), p<0.005 (***), p<0.0001 (****). Error bars represent standard error.