Received: 28 February 2018 | Accepted: 11 June 2018

DOI: 10.1111/cpr.12492

ORIGINAL ARTICLE

WILEY Cell Proliferation

AMOT130 linking F-actin to YAP is involved in intervertebral disc degeneration

Cong Zhang[1,2] | Feng Wang[1,2] | Zhiyang Xie[1] | Lu Chen[1] | Arjun Sinkemani[1] | Haomin Yu[1] | Xiaotao Wu[1,2]

[1]Department of Spine Surgery, Zhongda Hospital, School of Medicine, Southeast University, Nanjing, China

[2]Surgery Research Center, School of Medicine, Southeast University, Nanjing, China

Correspondence
Xiaotao Wu, Department of Spine Surgery, Zhongda Hospital, School of Medicine, Southeast University, Nanjing, China.
Email: wuxiaotaoispine@163.com

Funding information
Fundamental Research Funds for the Central Universities, Grant/Award Number: 2242016k40149, KYCX17_0182 and KYLX16_0302; Research Funds of Jiangsu Provincial Commission of Health and Family Planning, Grant/Award Number: H201533; National Natural Science Foundation of China, Grant/Award Number: 81572170, 81572190 and 81702203

Abstract

Objectives: Dysregulation of YAP by the Hippo signalling is associated with intervertebral disc degeneration (IDD). However, the relationship between the F-actin and Hippo pathway in IDD, and their effects on YAP remain poorly understood.

Methods: The characteristics of Hippo pathway and F-actin the in the NP (nucleus pulposus) and annulus fibrosus of immature, mature, ageing and disc degeneration model rats were observed by immunofluorescence, western blot and qPCR. Nucleus pulposus cells (NPCs) were transfected with lentivirus Sh-LATS A, Sh-LATS B and harvested for SA-β-gal staining, qPCR, western blotting and immunofluorescence staining to investigate the mechanism of Hippo pathway and F-actin interact in NPCs.

Results: We observed moderate decreases in F-actin and YAP expression with age in healthy intervertebral discs (IVDs). F-actin stress fibres distributed throughout the cytoplasm disappeared following treatment with latrunculin B (Lat B), resulting in a punctate distribution. Depletion of large tumour suppressor homologues 1/2 (LATS1/2) did not decrease the rate of cellular senescence, and YAP remained in the cytoplasm following Lat B treatment. Furthermore, angiomotin 130 (AMOT130) was associated with F-actin through a conserved actin-binding domain to retain YAP in the cytoplasm.

Conclusions: This study showed that a mechanism by which Hippo pathway and F-actin synergize to modulate YAP activation and localization in the context of IDD and help to control NPCs proliferation.

1 | INTRODUCTION

Intervertebral disc degeneration (IDD) is a common reason of lower back pain (LBP), affecting 70%-85% of all people at some point in their lives.[1] The aetiology of LBP is complex, with nociception driven by a combination of mechanical, inflammatory, and structural causes, of which 40% of cases are related to IDD.[2] LBP is generally considered a chronic disease, with current clinical strategies focused primarily on pain relief. These include conservative methods, such as treatment with nonsteroidal anti-inflammatory drugs, lumbar muscle functional exercise, rest and physical therapy; surgical interventions, including disc replacement, disc arthroplasty and spinal fusion; and biomedical engineering techniques, including biomaterial implantations, protein injections, gene transfers and other cell-based therapies.[3,4] However, these treatments have done very little to improve the physical, mental and social health of those affected. In recent years, novel strategies including growth factor injection, gene therapy and tissue engineering have been developed with the goal of promoting intervertebral disc (IVD) regeneration.[5] While these methods have shown modest success, to achieve full IVD regeneration it is necessary to restore the biological behaviour of resident cells and the surrounding

extracellular matrix (ECM), as well as preserve the biomechanical properties of healthy IVDs.

The IVD is an alymphatic and avascular tissue consisting of 3 distinct compartments: the gel-like nucleus pulposus (NP), cartilaginous endplates present on the superior and inferior surfaces, and the annulus fibrosus (AF), which can be further categorized into outer (OAF) and inner (IAF) layers.[6-8] The AF, in particular the OAF, withstands both tensional and strain forces, while the NP resists compressive loads applied to the spine. The NP is frequently implicated in IDD, and as such, has been the focus of much research into the pathophysiology and molecular mechanisms of IDD.

YAP has been found to be a key mechanotransducer, activated by a series of nuclear relays driven by mechanical stimuli.[9] YAP has been implicated in the regulation of organ size as part of the canonical Hippo signalling pathway, with their role in mechanotransduction mediated through noncanonical Hippo signalling. With their potent biological relevance in cellular mechanotransduction processes, the identification of the relationship between the Hippo signalling and cytoskeleton is becoming the leading edge in the field of mechanobiology.

Recently, some laboratories have sought to identify triggers of Hippo signalling activation. One such discovery is the control of YAP activity by the F-actin, which is not only required for cell morphological changes that accompany ECM binding and cell-cell adhesion but is also required for Hippo-mediated regulation of cell proliferation in both mammals and flies.[10-13] What is more, G-protein-coupled receptors could modulate Hippo pathway through F-actin. Specific processes include stabilization of F-actin, which leads to YAP activation, whereas disruption of F-actin, which in turn results in YAP inactivation.

Although several studies have implicated the cytoskeleton in mechanotransduction, the role of F-actin in the IVD remains poorly understood. In addition, the means by which nucleus pulposus cells (NPCs) sense their microenvironment, and the relationship and mechanism between YAP and F-actin in the development of IDD are not well understood. In our present study, we investigated the roles of Hippo signalling and F-actin in the NP and AF of immature (4 weeks), mature (14 weeks), ageing (50 weeks), and disc degeneration model (14 weeks which after annulus puncture at 4 weeks) rats. Furthermore, we describe the mechanism by which Hippo pathway and F-actin interact in NPCs.

2 | MATERIALS AND METHODS

2.1 | Animals and IDD analysis

Animal experiments were approved by the Institutional Animal Care and Use Committee in Southeast University School of Medicine. There are four groups in this study were analysed: a natural IDD group at 4 weeks (4w), 14 weeks (14w), and 50 weeks (50w) and a disc injury induced-IDD group (P4w). The 10-week-old rat coccygeal discs 5-9 were percutaneously punctured. Analyses were performed at 4 weeks (P4w) postoperatively, with the 14w group used as the P4w control. Forty male SD rats were randomly divided into 4w group, 14w group, P4w group and 50w group on average. IDD was analysed by X-ray, MRI and haematoxylin and eosin (HE) staining, as described previously.[8]

2.2 | Latrunculin B treatment

Nucleus pulposus cells were passaged three times (P3) in DMEM/F12 with 10% FBS, then plated into six-well plates at a density of 1×10^6 cells/well for 24 hours. Then the cells were treated with F-actin inhibitor latrunculin B (Lat B) for 1 hour. Stock solutions of Lat B was prepared at a final concentration of 2.5 mmol/L in DMSO, with working solutions diluted 1:1000 (2.5 μmol/L) in serum-free DMEM/F12. Control samples were treated with DMSO in serum-free DMEM/F12.

2.3 | SA-β-Gal staining

The passage 10 (P10) group consisted of senescent cells, as described previously.[8] NPCs were treated for 1 hour with Lat B, DMSO or lentivirus at a density of 1×10^6 cells/well, then stained with SA-β-Gal. Observation of cells under an inverted microscope, with NPCs senescence expressed as the percentage of senescent NPCs relative to total NPCs, analysed with Image-Pro Plus software.

2.4 | Immunofluorescence staining

The coccygeal 5-9 discs were dissected, fixed and decalcified in solution containing EDTA for 4 weeks at room temperature. The tissues (5 μm thickness) were incubated with anti-F-actin and anti-YAP antibodies. Sections were extensively washed by PBS three times and incubated with the Alexa Fluor 488-conjugated secondary antibody for 2 hours, washed, and mounted. Immunofluorescence analysis of NPCs was performed as described above following 1 hour of exposure to Lat B, DMSO or lentivirus.

2.5 | qPCR

Extraction of total RNA from NPCs and NP tissues using Trizol reagent. cDNA was synthesized and subjected to qPCR with genes primers in the presence of SYBR Green One according to previous study. Fold change of interested mRNA expression was normalized by GAPDH (Table 1).

2.6 | Western blot

NP tissue or NPCs protein samples were boiled at 95°C for 5 minutes and subjected to SDS-PAGE (10%). Then the proteins (40 μg) were separated and transferred onto a NC membrane. The membranes were incubated and were detected with primary

TABLE 1 Sequences of primers for qPCR

Gene		Primers
LATS1	Forward	ACCAGAAGACCGTCTAGGCA
	Reverse	TTCCTCGTTACCATCGCTCC
LATS2	Forward	ACCATGCTGCTGTTACTCCC
	Reverse	CGGTCTTCAGGGCTTCCTTT
YAP	Forward	ATTTCGGCAGGCAATACGGA
	Reverse	AGCTAATTCCCGCTCTGACG
CTGF	Forward	GGCGTAAAGCCAGGGAGTAA
	Reverse	CTCACTTCGGTGGGGTGTTT
GAPDH	Forward	ACAGCAACAGGGTGGTGGAC
	Reverse	TTTGAGGGTACAGCGAACTT

antibodies: p-YAP, YAP, LATS1, LATS2, F-actin, AMOT, CTGF and GAPDH (Table 2). Labels were quantified using the ImageJ software.

2.7 | Virus transfection

Lentivirus vectors contain the LATS1 and LATS2 knockdown (Sh-LATS A and Sh-LATS B) or a negative control sequence (Sh-Control) (GeneChem, Shanghai, China). Sequences of the Sh-LATS A and Sh-LATS B are listed in Table 3. Passage 2 NPCs were seeded in a six-well plates for 24 hours and then were transfected with different lentivirus vectors Sh-LATS A, Sh-LATS B or Sh-Control for 12 hours

TABLE 2 Primary antibodies

Antibody	Company (catalog number)
LATS1	Abcam (ab70562)
LATS2	Antibodies-online (ABIN753568)
YAP	Santa Cruz (sc-376830)
Phosphorylated YAP (Ser 127)	Abcam (ab172374)
CTGF	Abcam (ab6992)
F-actin	Abcam (ab205)
AMOT	Santa Cruz (sc-166924)
GAPDH	Bioss (bs-2188R)

TABLE 3 Sequences of lentivirus Sh-RNA

Sh-RNA	Sense strand sequence
LATS1 A	5′-caccGCCCAACAGGAACAGTCATAActcgagTTATG ACTGTTCCTGTTGGGC-3′
LATS2 A	5′-caccGGACCAAACAGTGACACTTCTttcaagagaAG AAGTGTCACTGTTTGGTCC-3′
LATS1 B	5′-caccGGGCATGAAATTCCTACATGGctcgagCCATG TAGGAATTTCATGCCC-3′
LATS2 B	5′-caccGCCTTGGAGTACATCAGTAAGttcaagagaCTT ACTGATGTACTCCAAGGC-3′
Control	5′-TTCTCCGAACGTGTCACGT-3′

(multiplication of infection = 20). After treatments, passage 3 NPCs were harvested for SA-β-gal staining, qPCR, western blotting and immunofluorescence staining.

2.8 | Laser scanning confocal microscopy assay

Nucleus pulposus cells were seeded in six-well plates at a density of 1×10^6 cells/well. After 24 hours, the NPCs were washed, fixed, permeabilized, blocked, and incubated AMOT130 and F-actin antibodies for 1 hour. Finally, DAPI was added and incubated for 10 minutes, then observed under laser scanning confocal microscopy.

2.9 | Coimmunoprecipitation assay

A spot of NPCs lysates (30 μL) harvested for western blot, remainder lysates (420 μL) was prepared for Co-IP assay. Protein A+G Agarose were incubated with AMOT130 or F-actin antibodies for 3 hours at 4°C under gently shake condition. Lysates was added to the beads prepared and incubated overnight at 4°C. Beads were washed by SDS protein loading buffer. Samples were boiled and performed by western blot.

2.10 | Statistical analyses

All results were expressed as mean ± SD. We performed statistical analysis using one-way ANOVA test. A value of $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | YAP and F-actin activated in acute annulus injury

IAF cells from the 4w group exhibited YAP was primarily in the cytoplasm in OAF cells, indicative of growth inhibition. By contrast, YAP mainly localized in the nucleus, indicative of cell growth and target gene transcription (Figure 1). Confocal microscopy revealed YAP distributed throughout the cytoplasm in cells isolated from NP, IAF (except 4w group) and OAF tissues in natural IDD rats, indicative of gradual degradation of NP tissues after 4 weeks of age. F-actin also exhibited modest decreases with age in natural IVD, as seen in the AF (IAF and OAF) and NP (Figure 2). By contrast, F-actin was upregulated in all areas of the disc (NP and AF), exhibiting a moderate punctate appearance in NPCs, but extending into the processes of OAF and IAF cells in the P4w group.

Under physiological conditions, the monomeric form G-actin is transformed into the polymeric form F-actin by ATP. In Western blot analyses, the P4w group exhibited YAP protein levels similar to that of F-actin, with results broadly consistent with that seen in IVD. Within these cells, F-actin was predominantly localized to the cell nucleus, with similar expression of both mRNA and protein. These results suggest that the acute damage seen in IVD upregulates the expression of both YAP and F-actin as part of the self-repair ability of the IVD (Figure 3). The purpose of self-repair of NP is to maintain

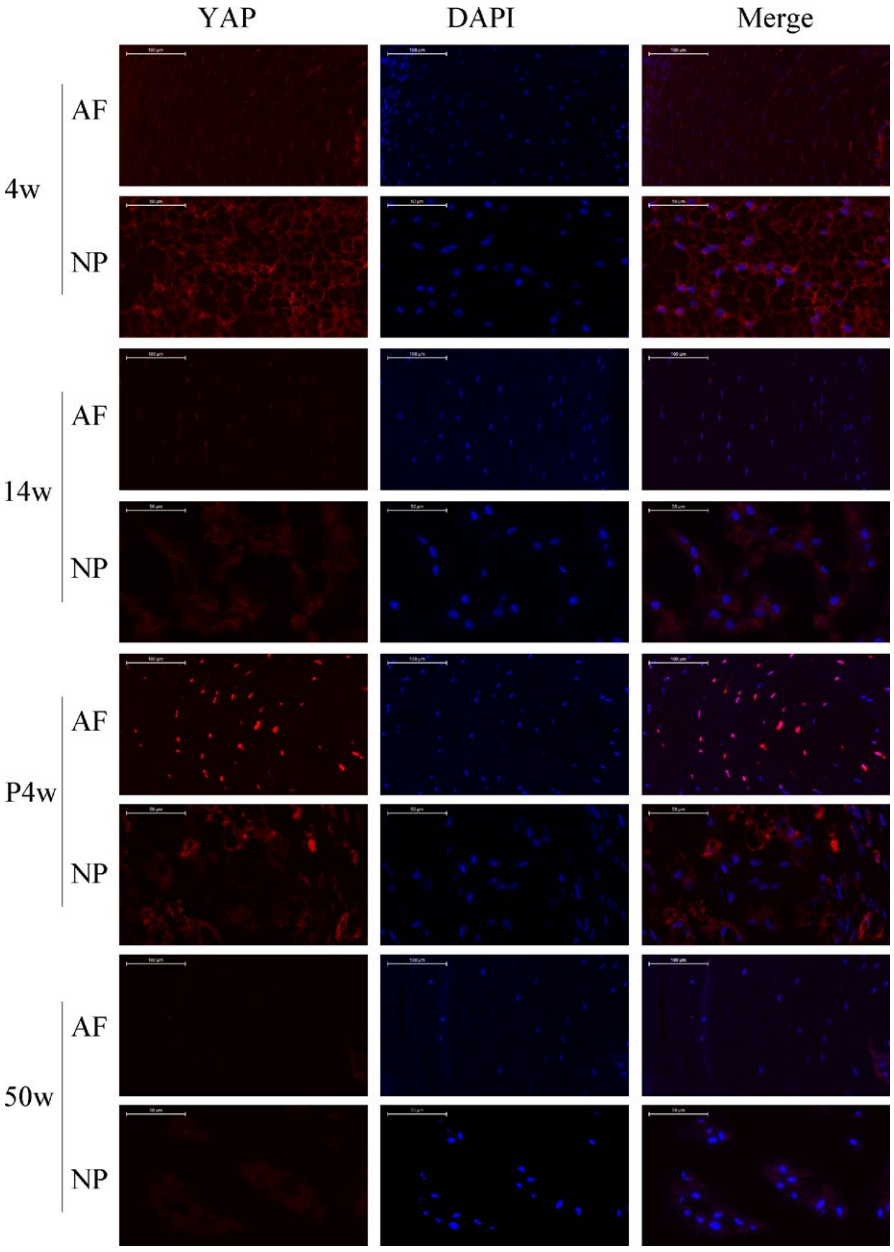

FIGURE 1 YAP levels decreased with age in the natural IVD group but were activated in acute disc injury

height and elasticity, while the purpose of AF is to prevent IVD protrusion. The final outcome is the enlargement of the area of the AF and the reduction of the area of the NP.

Based on examinations of NP tissue, the levels of upstream LATS1 increased in young rats, and YAP activity decreased with age (Figure 3). This LATS1-YAP regulation constitutes a negative feedback loop that maintains homeostasis of the Hippo signalling and prevents YAP overactivation. In the 50w group, LATS1 expression exhibited no obvious negative feedback relationship with YAP, which suggests that improper Hippo signalling may be a reason for IDD. Even more interesting is that in the P4w group, LATS1 decreased in response to increased YAP expression and decreased p-YAP expression, signifying the initiation of IVD self-repair. This observation is inconsistent with our previous findings in which CTGF mRNA expression was not correlated with protein abundance, indicative of incomplete compensation of the self-repair machinery, possibly as a result of posttranscriptional regulation.[8]

3.2 | Nuclear localization of YAP requires F-actin cytoskeleton integrity

To study the relationship between Hippo signalling, F-actin and YAP, we first examined the role of cytoskeletal integrity during YAP localization in the absence of NPCs. Cells were treated with pharmacological inhibitors, followed by immunolocalization of YAP and staining for F-actin using fluorescent phalloidin. In nonsenescent NPCs (P3), F-actin filaments were predominately localized beneath the cell membrane (Figure 4A); however, large numbers of F-actin stress fibres were observed throughout the cytoplasm and extending into the cellular protrusions. These fibres disappeared in response to Lat B

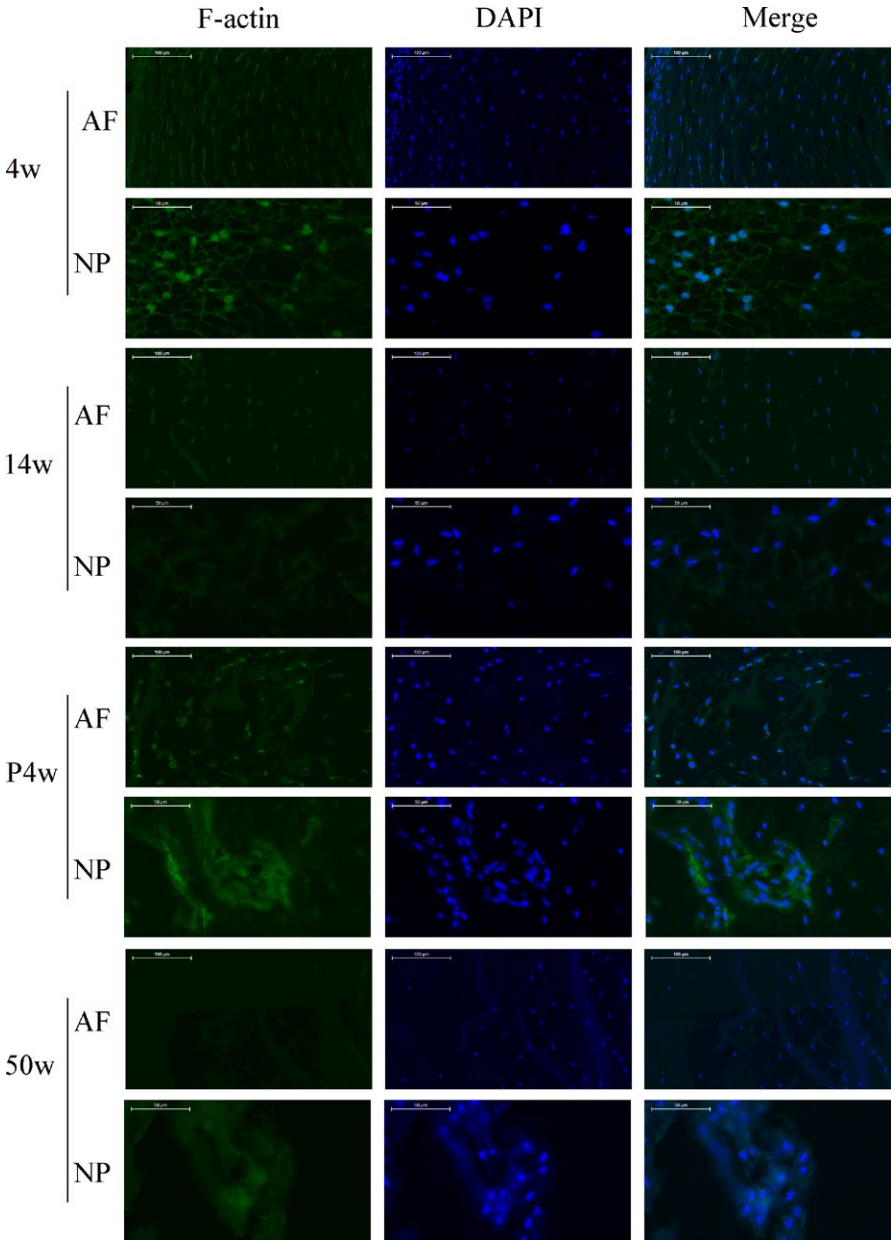

FIGURE 2 F-actin levels decreased with age in the natural IVD group but extended into the processes of OAF and IAF cells in the P4w group

treatment, reverting to a more punctate distribution, consistent with a depolymerization of F-actin (Figure 4A). These observations are consistent with those of Arupratan et al[14], who assessed actomyosin cytoskeleton dynamics in response to the myosin II ATPase inhibitor blebbistatin, showing that disruption of actomyosin did not affect YAP localization. Furthermore, the Hippo pathway was suppressed in the DMSO group, with YAP mainly localized in the NPCs nucleus indicating of downstream target gene transcription and cell growth (Figure 4B). By contrast, when F-actin was disrupted, cells became smaller, with YAP protein primarily sequestered in the cytoplasm.

3.3 | Inhibition of F-actin by Lat B significantly reduced proliferation, resulting in senescence of NPCs

Ki67 staining was reduced in cells treated with Lat B. The percentage of Ki67-positive cells in the DMSO group was ~40%, compared to only 10% after Lat B treatment (Figure 5A,B). To determine the effects of blocking F-actin on cell senescence, SA-β-Gal assay was used to measure differences in the Lat B and P3 groups (Figure 5C,D), revealing obvious senescence in NPCs after Lat B treatment. It is presumed that phenotypic differences between cell proliferation and senescence are caused by changes in YAP, as they were accompanied by decreases in YAP expression (Figure 5E-G).

3.4 | LATS-mediated regulation of YAP is blocked by F-actin inhibitor

Next, we investigated the effects of the cytoskeletal integrity and Hippo pathway on YAP nuclear localization and phosphorylation. To determine whether YAP localization was affected by LATS1/2, cells were treated with either Sh-Control or Sh-LATS1/2 to silence LATS gene expression.

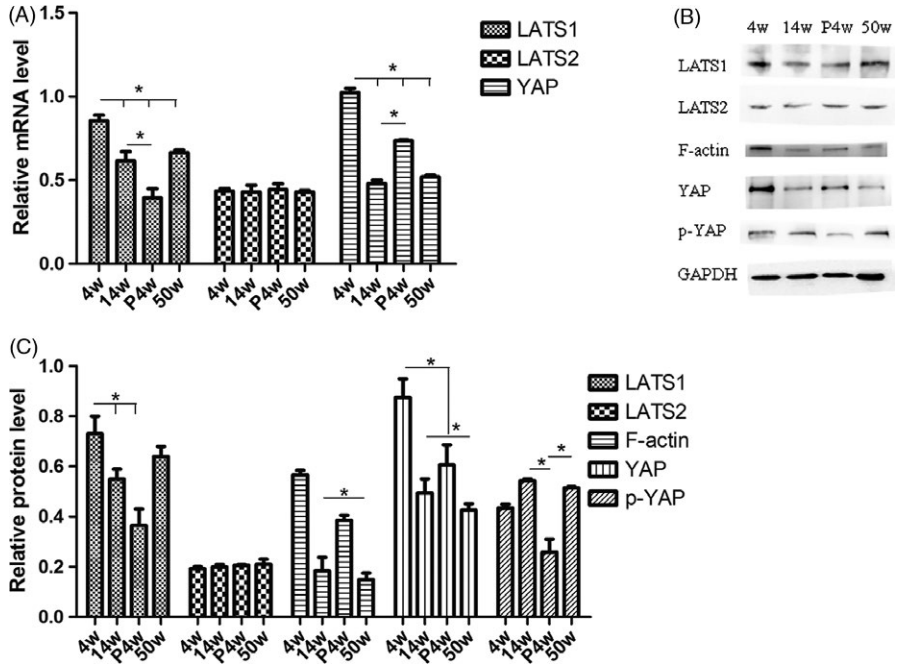

FIGURE 3 IDD occurred gradually in the natural IVD group, with F-actin expression consistent with that of YAP. (A) Relative mRNA levels of LATS1, LATS2 and YAP in the four groups (4w, 14w, P4w and 50w groups). (B, C) Hippo pathway and F-actin levels in the 4w, 14w, P4w and 50w groups as determined by Western blotting using YAP, F-actin, LATS1 and LATS2 primary antibodies

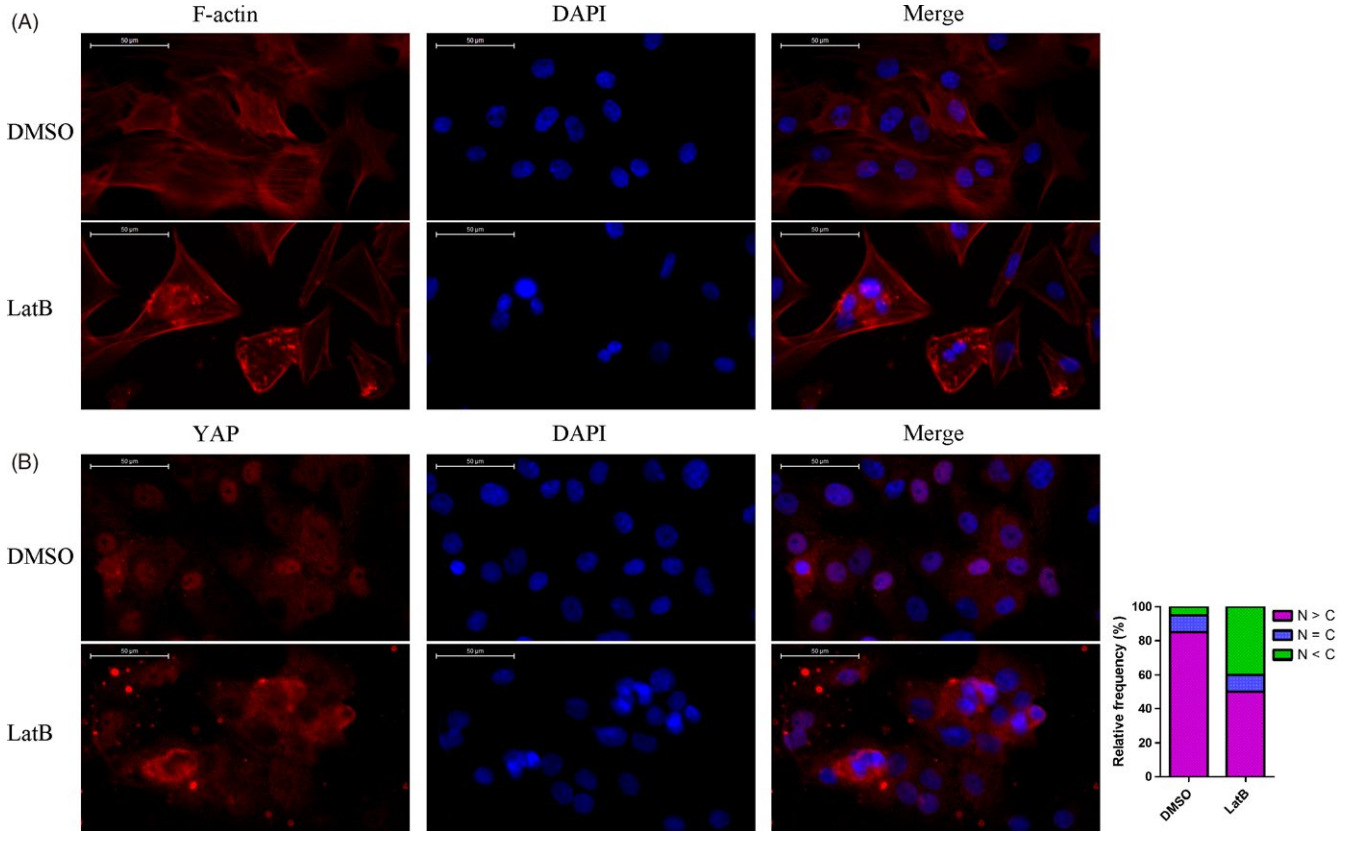

FIGURE 4 Nuclear localization of YAP requires F-actin cytoskeleton integrity. (A) F-actin stress fibres showed a mostly punctate distribution after Lat B treatment. (B) Hippo signalling was activated, with YAP primarily localized in the cytoplasm. (Right panel) Cells in five randomly fields were quantified for YAP subcellular localization. N, nuclear; C, cytoplasm

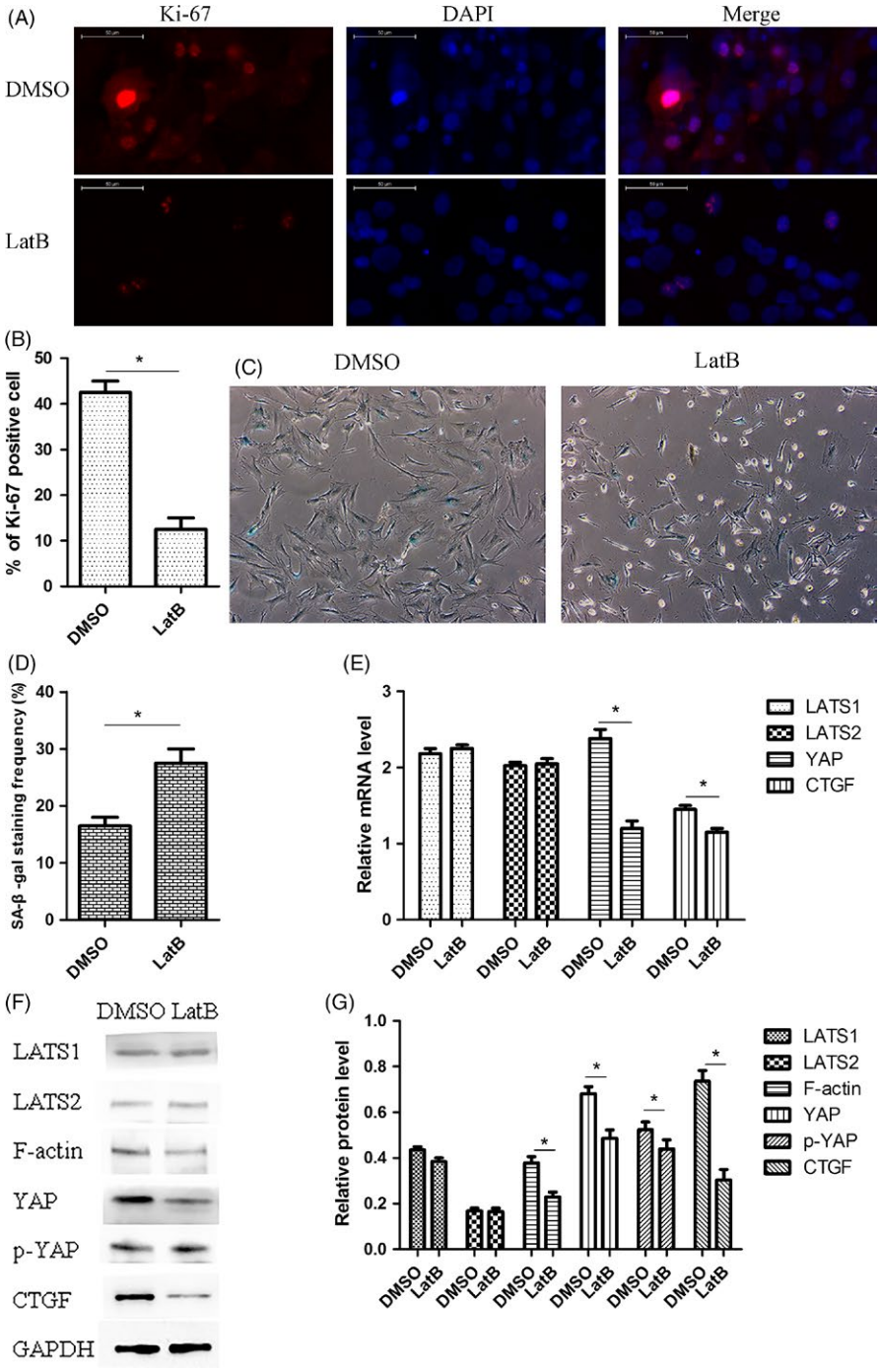

FIGURE 5 Inhibition of F-actin by Lat B significantly reduced proliferation and induced NPCs senescence. (A, B) Ki67 staining (A) and showing percentage of positive Ki67-stained cells (B). (C, D) Identification of senescent NPCs was determined based on SA-β-Gal activity, morphological observation, measurement of SA-β-Gal activity (C), and showing percentage of positive SA-β-Gal-stained cells (D). (E) Relative expression of LATS1, LATS2 and YAP in the DMSO and Lat B groups. (F, G) Western blot analysis of Hippo pathway-related proteins (anti-p-YAP, anti-YAP, anti-LATS1, anti-LATS2 and anti-CTGF antibodies) and F-actin expression

Unlike Lat B, which disrupted the peripheral F-actin band and excluded YAP from the nucleus, treatment with Sh-LATS1/2 led to greater nuclear accumulation of YAP. However, this effect was not found in Sh-LATS 1/2+Lat B group, with cells exhibiting a primarily punctate F-actin distribution, which suggests that F-actin integrity is essential for LATS-mediated regulation of YAP (Figure 6).

3.5 | LATS is a secondary regulator of YAP but LATS depletion inhibits YAP phosphorylation

Although the YAP and its downstream target gene CTGF increased in NPCs treated with Sh-LATS1/2, Ki67 and SA-β-Gal staining showed no increase in cell proliferation and senescence relative to controls (Figure 7A-D). One explanation may be the strong proliferation potential of low-passage NPCs; however, LATS1/2 depletion did not affect the senescence rate or YAP localization, which indicates that YAP is primarily regulated by F-actins other than LATS.

To determine the relationship between YAP phosphorylation and cytoskeletal perturbations, NPCs were treated with Sh-LATS1/2 or Sh-LATS1/2+ Lat B and assessed total YAP and YAP phosphorylation (p-YAP) levels by qPCR and Western blot (Figure 7E-G). Sh-LATS1/2 treatment decreased p-YAP (YAP activation), relative to controls, while p-YAP increased in response to Lat B. This observation is consistent with previous data detailing the effects of Lat A on YAP

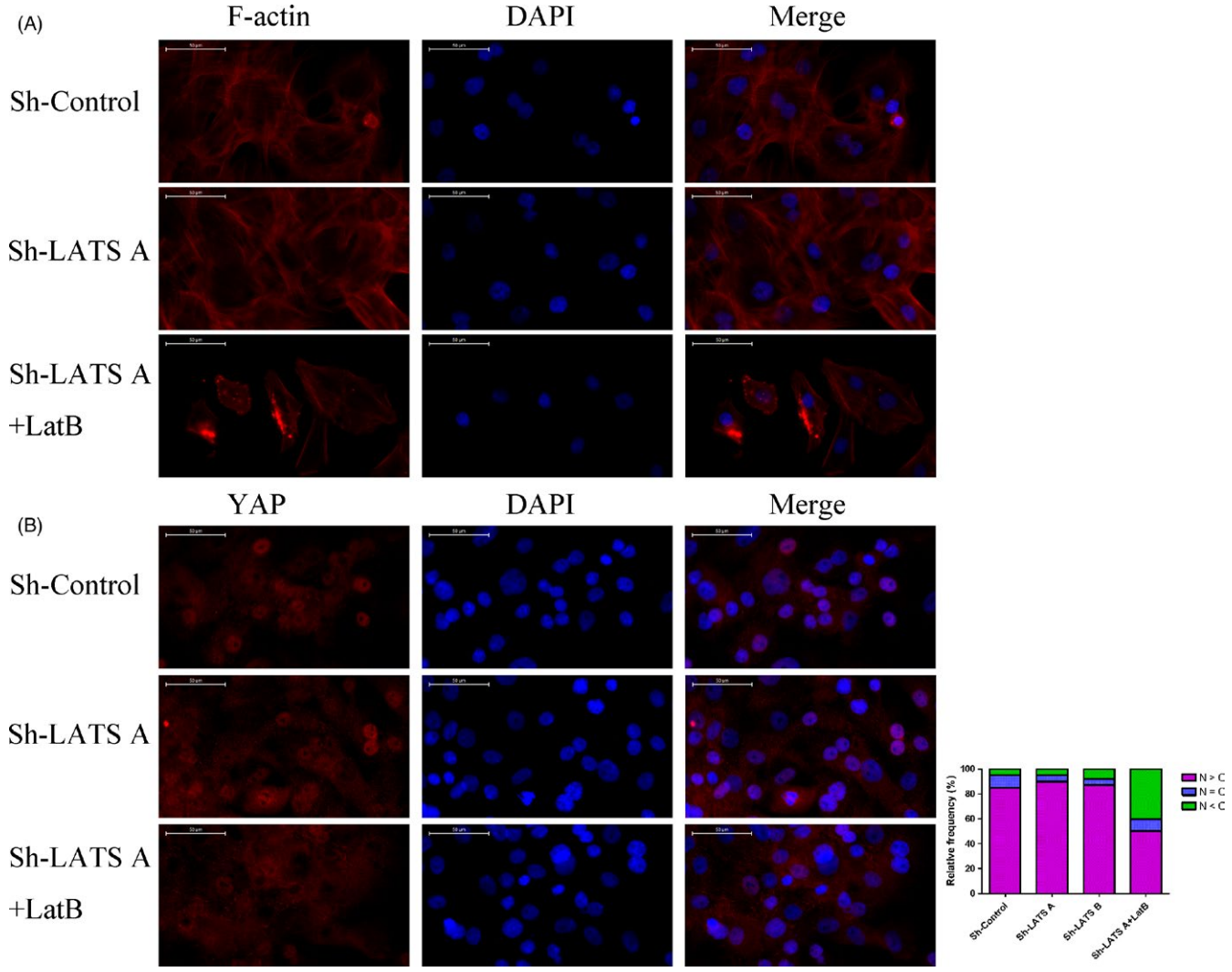

FIGURE 6 LATS1/2 inhibition induces nuclear accumulation of YAP. (A) F-actin stress fibres are primarily localized beneath the cell membrane. (B) Suppression of the Hippo signalling results in greater nuclear accumulation of YAP. (Right panel) Cells in five randomly fields were quantified for YAP subcellular localization. N, nuclear; C, cytoplasm

phosphorylation in embryonic fibroblasts of mice[14] and mammary epithelial cells.[15]

3.6 | F-actin and YAP compete for binding to AMOT130 and sequester YAP in the cytoplasm

Several recent studies have shown that YAP can bind to AMOT family proteins.[16-18] Binding of YAP to AMOT is mediated through its WW1 domain, which suggests that AMOT may restrict YAP transcriptional activity by competing for these binding sites. Therefore, we sought to examine changes in AMOT expression in relation to YAP, and to investigate the effects of these changes on interactions between F-actin and LATS. AMOT130 protein increased in response to disruption of F-actin by Lat B (Figure 8A,B) as well as LATS1/2 inhibition in NPCs, relative to the Sh-Control (Figure 8C,D).

To investigate the mechanism behind the decreased NPCs proliferation and increased senescence following the F-actin inhibition, and also observe whether F-actin and AMOT130 were interrelated, we examined for AMOT130 that interact with F-actin using Co-IP and co-localization. In the present study, the interaction between F-actin and AMOT130 was confirmed by CO-IP assay. What is more, laser scanning confocal microscopy showed that AMOT130 and F-actin were co-localized in NPCs. Competitive binding of F-actin and YAP to AMOT130 appears to underlie the ability of F-actin to prevent AMOT130 from binding and sequestering YAP in the cytoplasm.[19] Together these results suggest a model in which AMOT130 is sequestered on F-actin structures. However, when cells were treated with Lat B, leads to the release of AMOT130, allowing it to bind to and inhibit YAP.

4 | DISCUSSION

Hippo pathway activity is heavily influenced by extracellular hormones, such as epinephrine, glucagon, thrombin and sphingosine-1-phosphate.[20,21] These hormonal cues appear to be regulated

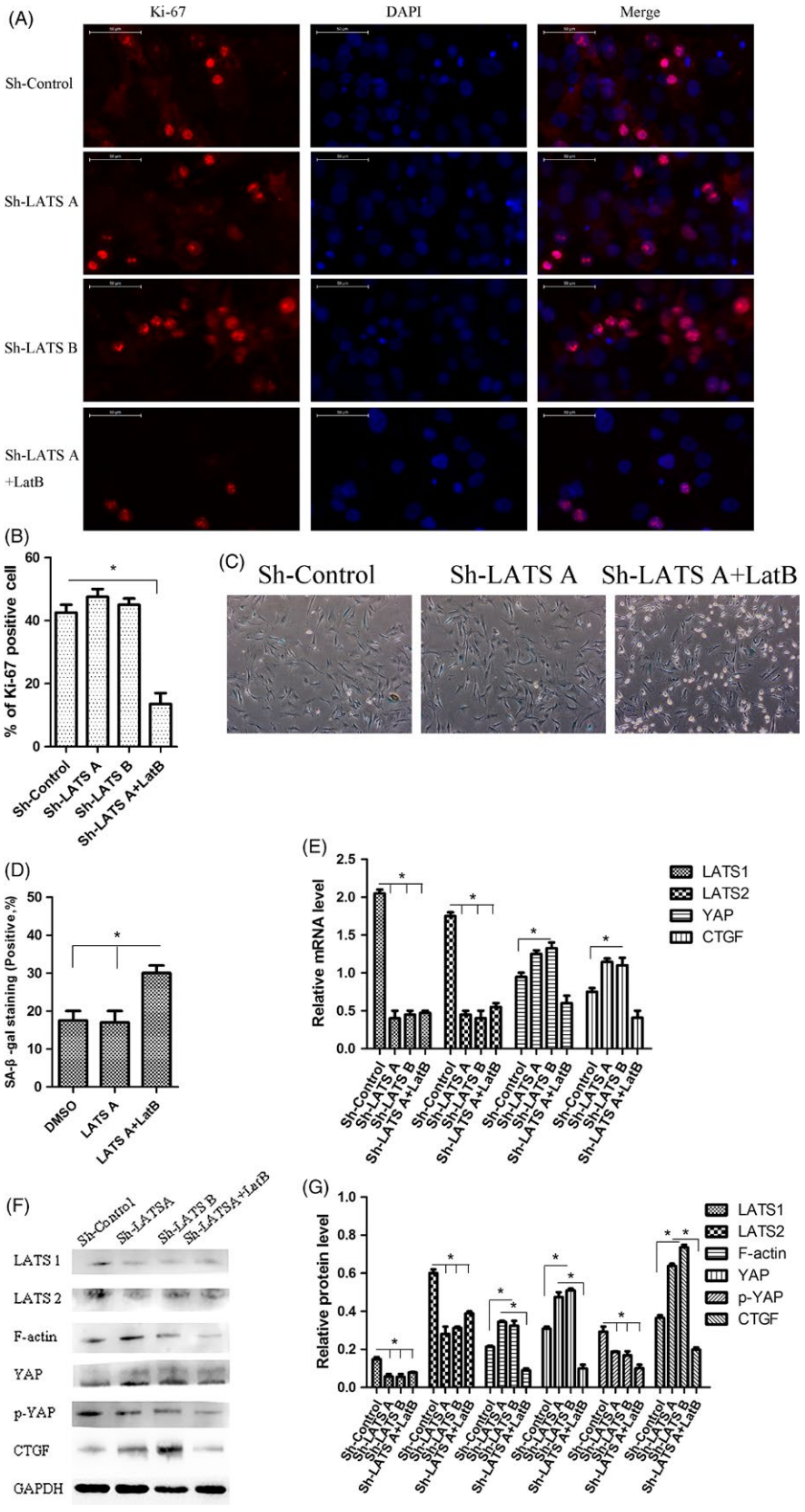

FIGURE 7 LATS1/2 depletion did not affect YAP localization in Lat B-treated cells. (A and B) Ki67 staining (A) and quantification of positive Ki67-stained cells (B). (C and D) Senescent NPCs was determined based on cell morphological observations and SA-β-Gal activity (C), and the percentage of positive SA-β-Gal-stained cells (D). (E) Relative expression of LATS1, LATS2 and YAP in the DMSO and Lat B groups by qPCR. (F and G) Western blot analysis of Hippo pathway-related proteins (anti-p-YAP, anti-YAP, anti-LATS1, anti-LATS2 and anti-CTGF antibodies) and F-actin expression

through the actin. In this study, YAP and F-actin decreased in association with age in both the AF and NP, which indicates a potential synergistic effect in the process of IDD. However, in acute disc injury, a strong correlation was seen between YAP and F-actin in IVD on both the mRNA and protein levels, with most expression localized in the nucleus, which indicates that acute damage to IVDs can induce

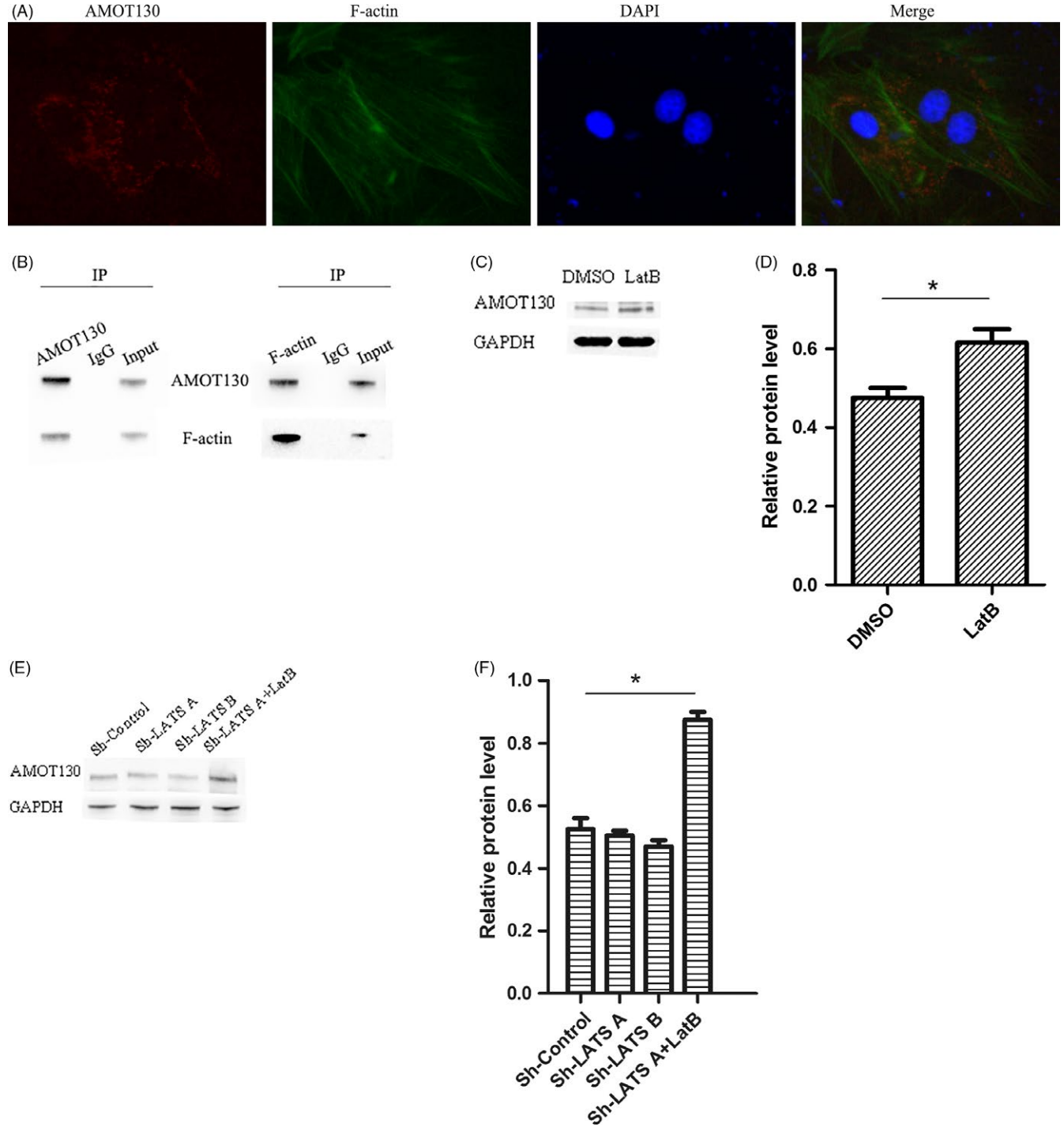

FIGURE 8 AMOT130 restricts YAP transcriptional activity by competing for F-actin binding. (A) Immunofluorescence staining showing F-actin and AMOT130 co-localized together in NPCs assessed with a laser scanning confocal microscope. (B) Coimmunoprecipitation demonstrates the interaction between F-actin and AMOT130. (C and D) Western blot analysis of AMOT protein expression in DMSO and Lat B-treated groups). (E and F) Western blot analysis of AMOT protein expression in the Sh-Control, Sh-LATS A, Sh-LATS B and Sh-LATS A + Lat B groups

the upregulation of the YAP and F-actin as part of the normal self-repair ability of the IVD.

LATS1 and 2 are direct targets of YAP, both of which increased upon YAP activation in this study. Oddly, no obvious changes in LATS2 expression were observed during the progression of natural IDD in rats. This suggests that LATS1 is the primary regulator of YAP, with little input from LATS2, although it is unclear if this effect extends beyond rats. In other studies, YAP has been shown to directly induce the transcription of neurofibromatosis type 2 (NF2), leading to YAP inhibition and LATS1 activation.[22,23] However, no obvious

negative feedback relationship was observed between LATS1 and YAP in the 50w group, which show that aberrant Hippo signalling may play a vital role in IDD progression.

Manipulation of F-actin expression through mechanisms such as inhibition of Rho by the C3 toxin or overexpression of Rho GTPases.[18,24,25] Rho GTPases and F-actin are key mediators of cellular signalling, activated by cell attachment, GPCR ligands and various mechanical cues. Not surprisingly, synthetic deletion of genes regulating the actin cytoskeleton has profound effects on YAP activity. For instance, knockdown of F-actin severing proteins (cofilin or gelsolin) or actin-capping proteins results in YAP activation.[26] Similarly, loss of the Capulet gene (inhibits actin polymerization) also leads to YAP activation and tissue overgrowth.[27]

Cytoskeleton proteins, including F-actin, directly influence cell morphology.[28] To evaluate the role of F-actin in YAP activity and localization, F-actin was disrupted by Lat B in normal cell cultures. Treatment of cells with Lat B leaded to nuclear YAP and decreased stress fibres. Wada et al[29] reported that cells treatment with the myosin inhibitors blebbistatin or Y27632, respectively, also nuclear YAP and decreased stress fibres. These results indicate that stress fibres consisting of F-actin are required for nuclear YAP. Furthermore, dysregulation of F-actin resulted in no significant changes in LATS1/2 expression relative to controls.

Treatment with cytochalasin D or Lat B disrupted F-actin, retaining YAP in the cytoplasm.[29,30] This retention was not blocked in cells by LATS1/2 knockdown, which is the dominant negative endogenous YAP. Intriguingly, although knockdown of LATS1/2 itself did not increase YAP downstream target genes in Lat B-treated NPCs, suggesting the actin cytoskeleton and LATS1/2 regulated YAP activity independently, which indicates that proper F-actin organization is a prerequisite for the function of LATS1/2. YAP and its downstream target CTGF increased in response to Sh-LATS1/2 in NPCs, resulting in YAP nuclear accumulation. However, cell proliferation and senescence were not significantly different than that of the Sh-Control group, which indicates that passage 3 NPCs still retain strong proliferative ability. Consistent with a recent in situ study, there were no obvious changes in LATS2 expression in cells treated with Lat B. This result was not surprising given the diverse responses of organs and cells types to Hippo signalling agonists. For instance, MST1/2, the core component of Hippo signalling, knockouts resulted in overgrowth of the stomach, heart, spleen and liver, but not the limbs or kidney.[31] Such a distinction is particularly likely in tissues such as breast and intestine, Where tissue-specific knockout of YAP did not induce any significant defects in tissue proliferation and morphology. The possible reason is that these tissues have MST1/2-independent regulators of YAP.

A major difference in our results compared to other studies is that mutating critical LATS1/2 phosphorylation residues did not block YAP inactivation by mechanical stress, and disruption of F-actin did not affect YAP phosphorylation.[15] More recent studies appear to contradict this finding, with evidence of LATS serving as an epistatic regulator of stress fibres, as well as F-actin regulating YAP through Hippo pathway, which acts either on LATS or upstream.[19,29,30,32] This discrepancy could be due to differences in cell lines as well as other differences in methodology.

Intriguingly, AMOT130 has also been shown to inhibition of YAP in a Hippo signalling independent manner by binding and sequestering it in the cytoplasm as well as through the activation of YAP inhibitory kinase LATS.[33,34] Which has been found to play a important role in the regulate of cell proliferation and organ size.[35,36] Our results confirmed that F-actin interacts with AMOT130 and AMOT130 increased in response to disruption of F-actin by Lat B. However, there has been questions regarding the importance of LATS for F-actin-dependent regulation of YAP.[15,26] Given the ability of LATS to associate with actin, we hypothesized that AMOT130 may mediate the effects of F-actin on YAP. Our work, together with other studies, suggest that angiomotins together with LATS1/2 to regulate YAP activity in response to F-actin disruption. We also show that AMOT130 regulates cytoplasmic retention of YAP after F-actin pertubation, with mutations in the AMOT that F-actin-binding motif increasing its ability to retain YAP in the cytosol. In the present study, AMOT130 interact with F-actin in NPCs and competitive binding of YAP and F-actin to AMOT130 appears to underlie the ability of F-actin to prevent AMOT130 from binding and sequestering YAP in the cytoplasm.

In summary, defects in the ability of YAP to localize to the cytoplasm in the context of F-actin disruption suggest a LATS1/2-dependent mechanism for YAP localization. In contrast, knockdown of LATS1/2 have a minor influence on YAP to regulate either cell shape or proliferation. Taken together, our results demonstrate that YAP activity can be inhibited through two distinct mechanisms: (i) the recruitment of YAP to the tight junction by direct AMOT130-YAP interactions, (ii) and YAP phosphorylation by the canonical Hippo signalling, which in turn promotes YAP cytoplasmic retention and inactivation by 14-3-3.

ACKNOWLEDGEMENTS

The research was supported by grants from the National Natural Science Foundation of China (81572170, 81572190, 81702203), the Fundamental Research Funds for the Central Universities (KYLX16_0302, KYCX17_0182, 2242016k40149), Research Funds of Jiangsu Provincial Commission of Health and Family Planning (H201533).

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/iafrL1.

CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Xiaotao Wu. Performed the experiments: Cong Zhang, Feng Wang, Zhiyang Xie, Lu Chen,

Arjun Sinkemani, Haomin Yu. Analysed the data: Cong Zhang, Feng Wang. Contributed the reagents/materials/analysis tool: Xiaotao Wu, Cong Zhang. Wrote the paper: Cong Zhang. All authors approved the final manuscript version for submittal.

ORCID

Xiaotao Wu http://orcid.org/0000-0002-7970-8911

REFERENCES

1. Andersson GB. Epidemiological features of chronic low-back pain. *Lancet*. 1999;354:581-585. https://doi.org/10.1016/s0140-6736(99)01312-4.
2. Schwarzer AC, Aprill CN, Derby R, Fortin J, Kine G, Bogduk N. The prevalence and clinical features of internal disc disruption in patients with chronic low back pain. *Spine*. 1995;20:1878-1883.
3. Cunha C, Lamas S, Goncalves RM, Barbosa MA. Joint analysis of IVD herniation and degeneration by rat caudal needle puncture model. *J Orthopaed Res*. 2017;35:258-268. https://doi.org/10.1002/jor.23114.
4. Iatridis JC, Nicoll SB, Michalek AJ, Walter BA, Gupta MS. Role of biomechanics in intervertebral disc degeneration and regenerative therapies: what needs repairing in the disc and what are promising biomaterials for its repair? *Spine J*. 2013;13:243-262.
5. van Uden S, Silva-Correia J, Oliveira JM, Reis RL. Current strategies for treatment of intervertebral disc degeneration: substitution and regeneration possibilities. *Biomater Res*. 2017;21:017-0106.
6. Xie ZY, Chen L, Wang F, et al. Endoplasmic reticulum stress is involved in nucleus pulposus degeneration and attenuates low pH-induced apoptosis of rat nucleus pulposus cells. *DNA Cell Biol*. 2017;36:627-637.
7. Wang F, Gao Z-X, Cai F, et al. Formation, function, and exhaustion of notochordal cytoplasmic vacuoles within intervertebral disc: current understanding and speculation. *Oncotarget*. 2017;8:57800-57812.
8. Zhang C, Wang F, Xie Z, et al. Dysregulation of YAP by the Hippo pathway is involved in intervertebral disc degeneration, cell contact inhibition, and cell senescence. *Oncotarget*. 2017;9:2175-2192.
9. Piccolo S, Dupont S, Cordenonsi M. The biology of YAP/TAZ: hippo signaling and beyond. *Physiol Rev*. 2014;94:1287-1312.
10. Hao J, Zhang Y, Wang Y, et al. Role of extracellular matrix and YAP/TAZ in cell fate determination. *Cell Signal*. 2014;26:186-191.
11. Li Y, Zhou H, Li F, et al. Angiomotin binding-induced activation of Merlin/NF2 in the Hippo pathway. *Cell Res*. 2015;25:801-817.
12. Mo JS. The role of extracellular biophysical cues in modulating the Hippo-YAP pathway. *BMB Rep*. 2017;50:71-78.
13. Budnar S, Yap AS. Versatile Scaffold: extrajunctional roles for alpha-Catenin. *Dev Cell*. 2017;43:381-382.
14. Das A, Fischer RS, Pan D, Waterman CM. YAP nuclear localization in the absence of cell-cell contact is mediated by a filamentous actin-dependent, myosin II- and phospho-YAP-independent pathway during extracellular matrix mechanosensing. *J Biol Chem*. 2016;291:6096-6110.
15. Dupont S, Morsut L, Aragona M, et al. Role of YAP/TAZ in mechanotransduction. *Nature*. 2011;474:179-183.
16. Liu Y, Lu Z, Shi Y, Sun F. AMOT is required for YAP function in high glucose induced liver malignancy. *Biochem Biophys Res Commun*. 2018;495:1555-1561.
17. Xiao J, Jin K, Wang J, et al. Conditional knockout of TFPI-1 in VSMCs of mice accelerates atherosclerosis by enhancing AMOT/YAP pathway. *Int J Cardiol*. 2017;228:605-614.
18. Shi X, Yin Z, Ling B, et al. Rho differentially regulates the Hippo pathway by modulating the interaction between Amot and Nf2 in the blastocyst. *Development*. 2017;144:3957-3967.
19. Mana-Capelli S, Paramasivam M, Dutta S, McCollum D. Angiomotins link F-actin architecture to Hippo pathway signaling. *Mol Biol Cell*. 2014;25:1676-1685.
20. Mo JS, Yu FX, Gong R, Brown JH, Guan KL. Regulation of the Hippo-YAP pathway by protease-activated receptors (PARs). *Genes Dev*. 2012;26:2138-2143.
21. Mo JS, Park HW, Guan KL. The Hippo signaling pathway in stem cell biology and cancer. *EMBO Rep*. 2014;15:642-656.
22. Chen Q, Zhang N, Xie R, et al. Homeostatic control of Hippo signaling activity revealed by an endogenous activating mutation in YAP. *Genes Dev*. 2015;29:1285-1297.
23. Dai X, Liu H, Shen S, et al. YAP activates the Hippo pathway in a negative feedback loop. *Cell Res*. 2017;25:1175-1178.
24. Yu FX, Zhao B, Panupinthu N, et al. Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. *Cell*. 2012;150:780-791.
25. Ding Z, Shi C, Jiang L, et al. Oncogenic dependency on beta-catenin in liver cancer cell lines correlates with pathway activation. *Oncotarget*. 2017;8:114526-114539.
26. Aragona M, Panciera T, Manfrin A, et al. A mechanical checkpoint controls multicellular growth through YAP/TAZ regulation by actin-processing factors. *Cell*. 2013;154:1047-1059.
27. Sansores-Garcia L, Bossuyt W, Wada K, et al. Modulating F-actin organization induces organ growth by affecting the Hippo pathway. *EMBO J*. 2011;30:2325-2335.
28. Liang F, Hwang JH, Tang NW, Hunziker W. Juxtanodin in retinal pigment epithelial cells: expression and biological activities in regulating cell morphology and actin cytoskeleton organization. *J Comp Neurol*. 2018;526:205-215.
29. Wada K, Itoga K, Okano T, Yonemura S, Sasaki H. Hippo pathway regulation by cell morphology and stress fibers. *Development*. 2011;138:3907-3914.
30. Zhao B, Li L, Wang L, Wang CY, Yu J, Guan KL. Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. *Genes Dev*. 2012;26:54-68.
31. Song H, Mak KK, Topol L, et al. Mammalian Mst1 and Mst2 kinases play essential roles in organ size control and tumor suppression. *Proc Natl Acad Sci U S A*. 2010;107:1431-1436.
32. Irvine KD, Shraiman BI. Mechanical control of growth: ideas, facts and challenges. *Development*. 2017;144:4238-4248.
33. Hirate Y, Hirahara S, Inoue K, et al. Polarity-dependent distribution of angiomotin localizes Hippo signaling in preimplantation embryos. *Curr Biol*. 2013;23:1181-1194.
34. Leung CY, Zernicka-Goetz M. Angiomotin prevents pluripotent lineage differentiation in mouse embryos via Hippo pathway-dependent and -independent mechanisms. *Nat Commun*. 2013;4:2251-2261.
35. Hong W. Angiomotin'g YAP into the nucleus for cell proliferation and cancer development. *Sci Signal*. 2013;6:2004573.
36. Dong J, Feldmann G, Huang J, et al. Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell*. 2007;130:1120-1133.

How to cite this article: Zhang C, Wang F, Xie Z, et al. AMOT130 linking F-actin to YAP is involved in intervertebral disc degeneration. *Cell Prolif*. 2018;51:e12492. -https://doi.org/10.1111/cpr.12492