Original Research

YAP signaling is involved in WDR1-regulated proliferation and migration of non-small-cell lung cancer cells

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Impact Statement

Our study is the first to explore the effect of WD-repeat domain 1 (WDR1) on YAP (Yes-associated protein) signaling. Our study demonstrates the role of WDR1 in regulating YAP signaling in non-small-cell lung cancer (NSCLC) cells. WDR1 and YAP may be valuable biomarkers of aggressiveness and poor prognosis in NSCLC. Disruption of actin dynamics and inhibition of the YAP signaling pathway may be potential targets of therapeutic strategies for NSCLC and provide clues for the molecular-level treatment of NSCLC.

Abstract

As a major co-factor of F-actin depolymerization, WD-repeat domain 1 (WDR1) affects the cellular microenvironment by cytoskeleton remodeling, thereby influencing cell molecular behavior. Our previous study showed that WDR1 activates YAP (Yes-associated protein) signaling in non-small-cell lung cancer (NSCLC) cells, but the mechanism remains unclear. We discovered that knockdown WDR1 in NSCLC cells decreased the expression of YAP and the nucleus-to-cytoplasm ratio. Disruption of cortical stress by drugs significantly inhibited YAP nuclear trafficking and enhanced YAP phosphorylation. In WDR1-knockdown NSCLC cells, inhibition of Hippo pathway reduced the nuclear exclusion of YAP and phosphorylated YAP. Our data suggest that WDR1-mediated cortical stress might be involved in regulating YAP signaling, thereby affecting the proliferation and migration of NSCLC cells.

Keywords: WDR1, YAP, non-small-cell lung cancer, cytoskeleton

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Introduction

Actin cytoskeleton and cell mobility are crucial for cancer genesis and progression.[1] Cytoskeleton-mediated cell tension is indispensable for regulating Yes-associated protein (YAP) nuclear translocation.[2] Actin-depolymerizing factor (ADF)/cofilin is an essential regulator of actin dynamics that promotes actin disassembly by severing actin filaments and dissociating actin monomers from actin filaments.[3] WD-repeat domain 1 (WDR1) (the mammalian homolog of actin interacting protein 1 in *Drosophila*) is an ADF/cofilin's co-factor that accelerates the depolymerization of ADF/cofilin-bound actin filaments.[4,5] WDR1 plays important roles in many actin-associated biological processes, such as cell migration and morphology.[6] WDR1 is necessary for cell capacity to generate/maintain cortical tension; WDR1-depleted mouse epidermis led to morphological and epidermal tension abnormalities.[7] Previously, we showed that WDR1 influenced YAP phosphorylation in the A549 cell line.[6] It had been showed that YAP signaling can be regulated by Hippo pathway and the actin cytoskeleton.[8,9] However, the molecular mechanism that WDR1 modulates YAP signaling is still unknown.

YAP and transcriptional co-activator with PDZ-binding motif (TAZ) play vital roles in regulating organ growth,[10] regeneration,[11,12] and cellular plasticity.[13] YAP/TAZ is aberrantly expressed in many human tumors and have been shown to be critical for cancer initiation,[14,15] development, or metastasis.[16,17] YAP/TAZ expression is higher in cancer tissues than in normal tissues, which is very attractive for targeted therapy in clinical practice.

As a canonical mechanism that regulates YAP/TAZ, phosphorylation cascade of the Hippo pathway had been identified by Boopathy and Hong.[18] The core kinases involved in the Hippo signaling pathway are mammalian Ste20-like kinases 1/2 (MST1/2), Salvador homolog 1 (SAV1), Mps one binder (MOB) kinase activator 1 (MOB1), and large tumor suppressor 1/2 (LATS1/2).[19] Phosphorylation of MST1/2 phosphorylates MOB1, which then phosphorylates LATS1/2.[20] Subsequently, the effector YAP is phosphorylated

and retained and degraded in the cytoplasm.[21,22] When the Hippo pathway is blocked, unphosphorylated YAP translocates to the nucleus, where it forms a complex with DNA-binding transcription factors, including TEA-domain transcription factor (TEAD), *Drosophila* mothers against decapentaplegic proteins (Smads), and T-box transcription factor 5 (TBX5).[23,24]

Here, we have shown that WDR1-depleted cells exhibited both cellular morphology and actin cytoskeleton disruption and decreased activity of YAP signaling. Moreover, cells treated with blebbistatin (a myosin II inhibitor) and Y27632 (a selective inhibitor of Rho-associated) to disrupt the cytoskeleton showed similar phenotypes to WDR1 knockdown. Furthermore, the increased nucleus-to-cytoplasm (N/C) ratio of YAP, increased cell proliferation and migration, and the endogenous target genes of YAP signaling in WDR1-over-expressed A549 cells were disrupted upon Y27632 and blebbistatin treatments. Our data suggested that WDR1-regulated YAP signaling might occur through actin dynamics-mediated cortical tension.

Knockdown of LATS1/2 showed that WDR1-regulated YAP signaling is partially Hippo-dependent. According to our findings, the WDR1−YAP axis could become a potential therapeutic target for NSCLC.

Materials and methods

Cell culture and transfection

Human NSCLC cell line A549 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI-1640 (Meilunbio, Dalian, China) with 10% fetal bovine serum (FBS) was used for cell culture. The lentiviral vector for pLKO.1-TRC was used to construct the short hairpin RNA (shRNA) expression plasmid. The shRNA sequence of knockdown WDR1 (shWDR1) is 5′-GCTGGGAAGATCAAAGACATT-3′, and the shRNA sequence of the control group (shCTL) is 5′-GCAAGCTGACCCTGAAGTTCAT-3′. WDR1 cDNA was cloned into the PCDH-Flag lentiviral vector as a positive control to create a cell line with stable WDR1 over-expression, whereas PCDH-GFP was utilized as a negative control. Finally, puromycin was used to screen for transduced cells. The siRNA (GenePharma, Shanghai, China) sequences targeting LATS1 and LATS2 were GCAAGUCACUCUGCUAATT and GGAAGAUCCUCUACCAGAATT. RNAs (20 nM) mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were transfected into A549 cells. The cells were then grown for 48 h before being analyzed.

Western blotting

A549 cells were used to extract total cell protein, and the protein concentration was measured by the bicinchoninic acid (BCA) assay kit (Beyotime Biotechnology, Shanghai, China). The NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific, Waltham, MA, USA) was used to separate nucleus and cytoplasm proteins according to the manufacturer's instructions. The protein levels were then determined by western blotting as reported before.[6] Protein was transferred to the polyvinylidene difluoride (PVDF) membrane and incubated with the primary antibody overnight at 4°C. The antibodies used for incubation were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (AP0063; Bioworld Technology, Nanjing, China), WDR1 (13676-1-AP; Proteintech Group, Wuhan, China), TATA-box-binding protein (TBP) (Ab818; Abcam, Cambridge, England), pYAP (S397) (13619; Cell Signaling Technology, Darmstadt, Germany), YAP (Ab52771; Abcam), pYAP (S127) (13008S; Cell Signaling Technology), and cofilin (BS2183; Bioworld Technology). After incubating with horseradish peroxidase (HRP)-labeled anti-mouse or anti-rabbit IgG for 1 h at room temperature, the target protein was detected by visual chemiluminescence.

Luciferase reporter assay

In order to evaluate the activity of YAP, luciferase activity was measured. The plasmid 8xGTIIC-Luc is described elsewhere.[25–27] The luciferase reporter plasmid 8xGTIIC was transfected into A549 cells with over-expressing or knocking down WDR1. The Dual-Luciferase Reporter Gene Assay Kit (Promega, Madison, Wisconsin, USA) was used to measure luciferase activity in cellular extracts.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted from the cells using RNAex Pro Reagent (AG21101; Accurate Biotechnology, Changsha, China). Each specimen had one microgram of total RNA reverse-transcribed to cDNA using the Vazyme (R222-01) Reverse Transcription Kit (Vazyme Biotech Co. Ltd. Nanjing, China). AceQ qPCR SYBR Green Master Mix (Q511-02) was used to perform quantitative real-time polymerase chain reaction (qPCR) on a CFX96 PCR instrument (Bio-Rad, Berkeley, CA, USA). Primer Premier 5.0 was used to create all of the primers. The primers used in this work were for CTGF, 5′-CAGCATGGACGTTCGTCTG-3′ (forward) and 5′-AACCACGGTTTGGTCCTTGG-3′ (reverse); for cysteine-rich angiogenic inducer 61 (CYR61), 5′-CTCGCCTTAGTCGTCACCC-3′ (forward) and 5′-CGCCGAAGTTGCATTCCAG-3′ (reverse); for ANKRD1, 5′-AGTAGAGGAACTGGTCACTGG-3′ (forward) and 5′-TGTTTCTCGCTTTTCCACTGTT-3′ (reverse); for GAPDH, 5′-TGTGGGCATCAATGGATTTGG-3′ (forward) and 5′-ACACCATGTATTCCGGGTCAAT-3′ (reverse); for LATS1, 5′-AATTTGGGACGCATCATAAAGCC-3′ (forward) and 5′-TCGTCGAGGATCTTGGTAACTC-3′ (reverse); for LATS2, 5′-ACTTTTCCTGCCACGACTTATTC-3′ (forward) and 5′-GATGGCTGTTTTAACCCCTCA-3′ (reverse). GAPDH was used to standardize the relative messenger RNA (mRNA) levels. The $\Delta\Delta C_t$ value was calculated in accordance with the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was detected using the Cell Counting Kit-8 (CCK8; Boster, Wuhan, China). Each experiment was carried out five times in a row.

Clonal formation assay

Cells subjected to various treatments were plated into 10 mm × 20 mm cell culture dishes (2000 cells) and grown in RPMI1640 media containing 10% FBS for 10 days. After phosphate-buffered saline (PBS) washing, the plates were then stained with crystal violet. Digital photographs were then obtained as a basis for analysis.

Wound healing assay

The cells were starved for 12 h after being implanted onto six-well plates. When the cells had attained 90–100% confluence, the cell layer was scratched using a sterilized 200-μL pipette tip. At 0, 24, 48, and 72 h after scratching, photographs were captured using an inverted microscope with a camera attached (Olympus, Tokyo, Japan). Magnification of 100× was used to capture the images. Each experiment was repeated three times independently.

Immunofluorescence staining

Cells exposed to different treatments were seeded on coverslips in 12-well plates and then fixed in 4% paraformaldehyde for 20 min at room temperature. Cells were permeabilized in 0.2% Triton X-100 for 5 min. To detect actin, the cells were stained for 30 min at room temperature with (TRITC)-conjugated phalloidin (p1951; Sigma-Aldrich, St. Louis, Missouri, USA) and 4,6-diamidino-2-phenylindole (DAPI) (D6584; Sangon Biotech, Shanghai, China).

Statistical analyses

For statistical analysis, the unpaired two-tailed Student's *t* test was utilized. The results were provided as mean ± SEM. $P < 0.05$ was considered significant.

Results

WDR1 affected the activity of YAP

Our previous data showed that WDR1 knockdown in NSCLC cells (A549) significantly reduced the protein levels of YAP.[6] However, the mechanism of WDR1-dependent YAP regulation has not yet been clarified. Here, we used shRNA to create stable WDR1-knockdown cells. Consistent with previous data,[6] western blot and immunofluorescence analyses revealed that knocking down WDR1 (shWDR1) significantly reduced the level of total YAP, increased the inhibitory phosphorylation of YAP (Figure 1(a)), and decreased the N/C ratio of YAP, as shown in Figure 1(b) to (d). Next, we over-expressed WDR1 in A549 cells. Western blot analysis revealed that over-expressed WDR1 increased the level of total YAP, decreased the phosphorylation of YAP (Figure 1(e)), and increased the N/C ratio of YAP (Figure 1(f)). These results suggested that WDR1 affected the phosphorylation and cytoplasmic distribution of YAP. Surprisingly, the mRNA level of YAP was not dramatically altered (Figure 1(g)). Therefore, we hypothesized that the WDR1-dependent YAP regulation was at the level of post-transcription, the detailed mechanism of which is still unclear.

As an important transcriptional regulator for cell growth and development, YAP regulates cell proliferation and migration by modulating TEAD transcriptional activity (Figure 1(h)). To determine how WDR1 affected YAP signaling, we measured the transcriptional activity of TEAD by a luciferase activity reporter plasmid with a TEAD-binding site (8×GT-IIC-Luc). WDR1 over-expression significantly increased the transcriptional activity of TEAD, and depletion of WDR1 decreased the transcriptional activity of TEAD (Figure 1(h)). Altogether, these results suggested that WDR1 positively regulates YAP–TEAD-driven transcription.

Cortical tension might involve regulation of YAP signaling mediated by WDR1

The ability of eukaryotic cells to resist deformation, material transport, and morphological changes during movement depends on the cytoskeleton.[28] The cytoskeleton is affected by both internal and external physical pressures, altering local mechanical characteristics and cell behavior.[29] Previously, our study showed that WDR1-depleted cells displayed a rounder cell shape, shortened cellular protrusion, and abnormal distribution of F-actin.[6] WDR1 is reported to be required for cells to generate/maintain cortical tension.[7] We performed experiments to examine whether WDR1 regulates YAP signaling via cortical tension.

We over-expressed cofilin in WDR1-knockdown A549 cells (Figure 2(a)). We noted that over-expression of cofilin rescued the N/C ratio of YAP, F-actin cumulations, and the inhibition of migration and proliferation in WDR1-knockdown cells (Figure 2(b) to (f)). These results indicated that WDR1-regulated YAP signaling is dependent on cofilin-mediated actin dynamics. Cytoskeletal tension is reported to affect the nuclear localization and activity of YAP/TAZ.[30] Therefore, we hypothesized that knocking down WDR1 would affect the nuclear localization and activity of YAP by altering the cytoskeletal tension. We treated cells with blebbistatin (a non-muscle myosin type II ATPase inhibitor) and Y27632 (ROCK inhibitor) to disrupt the cortical tension, which mimics the effect of loss of WDR1 (shWDR1) on YAP. As shown in Figure 3(a), Y27632 and blebbistatin treatment for 6 h resulted in disrupted stress fibers and a stack-like F-actin phenotype (Figure 3(a)). In blebbistatin- and Y27632-treated A549 cells, the protein level of total YAP decreased by 2.0 and 1.6 times, respectively, and phosphorylated YAP levels increased significantly (Figure 3(b)). Furthermore, the nucleo-cytoplasmic separation result also showed that disruption of the F-actin cytoskeleton resulted in reduced nuclear YAP (Figure 3(c)). Consistent with the results of WDR1 knockdown, Y27632 and blebbistatin significantly inhibited the migration and proliferation of A549 cells (Figure 3(d) to (f)).

To further clarify the function of cortical tension in WDR1-regulated YAP signaling, we over-expressed WDR1 in A549 cells (Figure 4(a)). WDR1 over-expression reduced YAP phosphorylation while increased YAP N/C ratio (Figure 4(b) and (c)). Furthermore, the increased N/C ratio of YAP (Figure 4(c)) increased cell proliferation and migration, and the endogenous target genes of YAP signaling

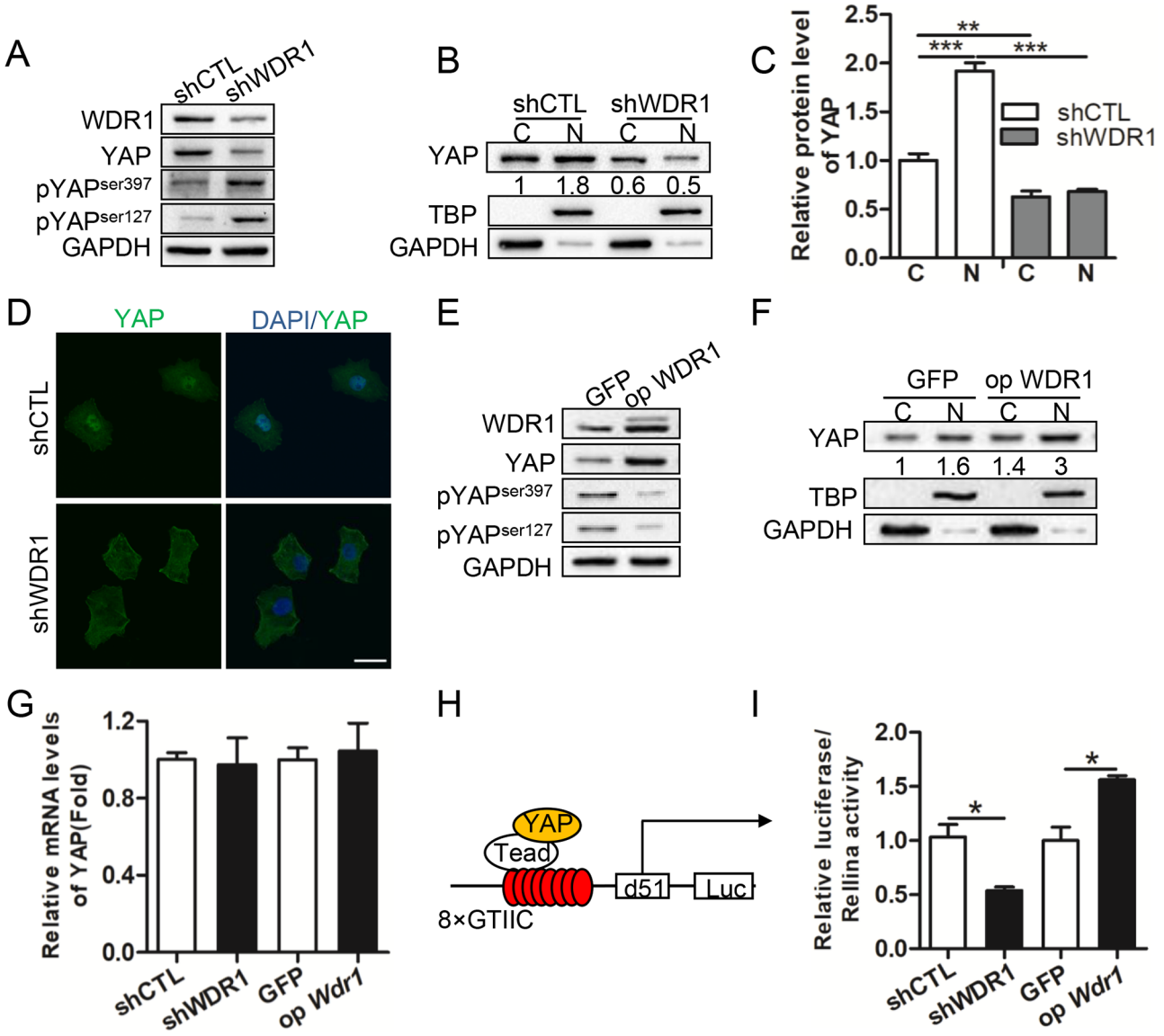

Figure 1. Effects of WDR1 on the transcriptional activity of YAP/TAZ. (a) Stable WDR1 knockdown in A549 cell lines were generated by shRNA. shWDR1 significantly reduced WDR1 expression levels. WDR1 depletion reduced the expression level of YAP and increased the level of pYAP (S397) and pYAP (S127). (b) WDR1 knockdown reduced the N/C ratio of YAP. The quantified protein levels were shown beneath the corresponding bands. (c) Nucleo-cytoplasmic separation experiments were conducted three times; the relative protein level was quantified. (d) Immunofluorescence staining analysis of YAP in A549-shCTL and A549-shWDR1 cells. Cells were fixed and stained with the indicated antibodies for YAP. Cell nuclei were stained with DAPI. (e) To over-express WDR1, the eukaryotic expression vectors were transfected into cells, and western blot analysis indicated that WDR1 was effectively increased in the WDR1 group. WDR1 over-expression enhanced the expression level of total YAP and decreased the levels of pYAP (S397) and pYAP (S127). The quantified protein levels were shown beneath the corresponding bands. (f) WDR1 over-expression enhanced the N/C ratio of YAP. The quantified protein levels were shown beneath the corresponding bands. (g) qPCR was used to detect the mRNA levels of YAP. (h) Schematic presentation of the TEAD-reporter plasmid used in this study. (i) The luciferase reporter assay's results. GTIIC was the TEAD-binding motif. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

in WDR1-over-expressed A549 cells were interfered upon Y27632 and blebbistatin treatments (Figure 4(d) to (h)). Our results suggested that WDR1 regulated YAP signaling might by cytoskeleton-mediated cortical tension.

Hippo-dependent YAP signaling contributed to WDR1-regulated migration and proliferation

Typical Hippo signaling in vertebrate cells involves a cascade of two vital serine/threonine kinases (MST1/2 and LATS1/2).[31] Activated LATS1/2 phosphorylates the transcriptional co-activators YAP and TAZ, the two main downstream effectors of the Hippo signaling pathway.

To clarify whether WDR1-regulated YAP signaling is Hippo signaling pathway-dependent, we performed LATS1/2 knockdown in shWDR1 cells (Figure 5(a) to (c)). Western blot analysis showed a significant decrease in the level of phosphorylated YAP after LATS1/2 knockdown (Figure 5(d)). In addition, the total YAP also recovered comparison with the shWDR1 group. The nucleo-cytoplasmic separation findings revealed that knockdown of LATS1/2 increased the level of YAP in the nucleus compared to the

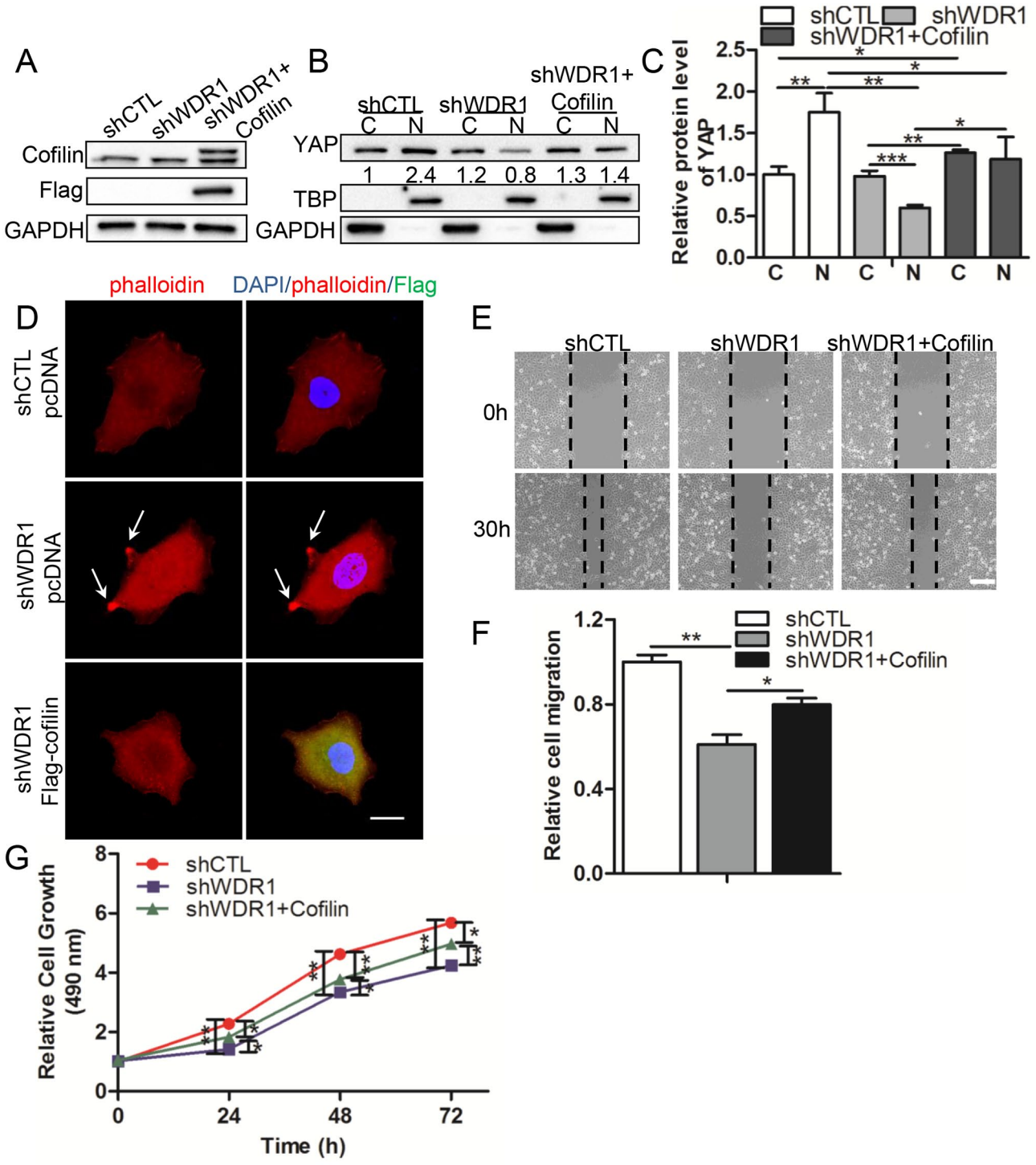

Figure 2. YAP signaling was regulated by WDR1-mediated actin dynamics. (a) Cofilin over-expression in shWDR1 cells. The efficiency of cofilin over-expression was determined by western blotting. (b) Western blot analysis showed over-expression of cofilin significantly increased the N/C ratio of YAP. The quantified protein levels were shown beneath the corresponding bands. (c) The quantification results of western blotting in (b). Nucleo-cytoplasmic separation experiments were conducted three times. (d) Phalloidin and cofilin (anti-Flag) immunofluorescence staining. Over-expression of cofilin rescued F-actin accumulation caused by WDR1 depletion. F-actin accumulations were indicated by the arrow. Scale bar = 20 μm. (e) and (f) Wound healing assay for A549 cells with different treatments. Knockdown of WDR1 inhibits the migration of A549 cells, but cofilin over-expression partially rescued the migration inhibited by WDR1 knockdown. Scale bar = 200 μm. (g) CCK8 assay was used to detect the proliferation of A549 cells. Knockdown of WDR1 inhibited the proliferation of A549 cells, but over-expression of cofilin dramatically improved the capacity of proliferation in A549 cells. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

shWDR1 group (Figure 5(e)). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) results demonstrated that in LATS1/2-knockdown shWDR1 cells, the expressions of endogenous target genes, including connective tissue growth factor (CTGF), ankyrin repeat domain-containing protein 1 (ANKRD1), and CYR61 were

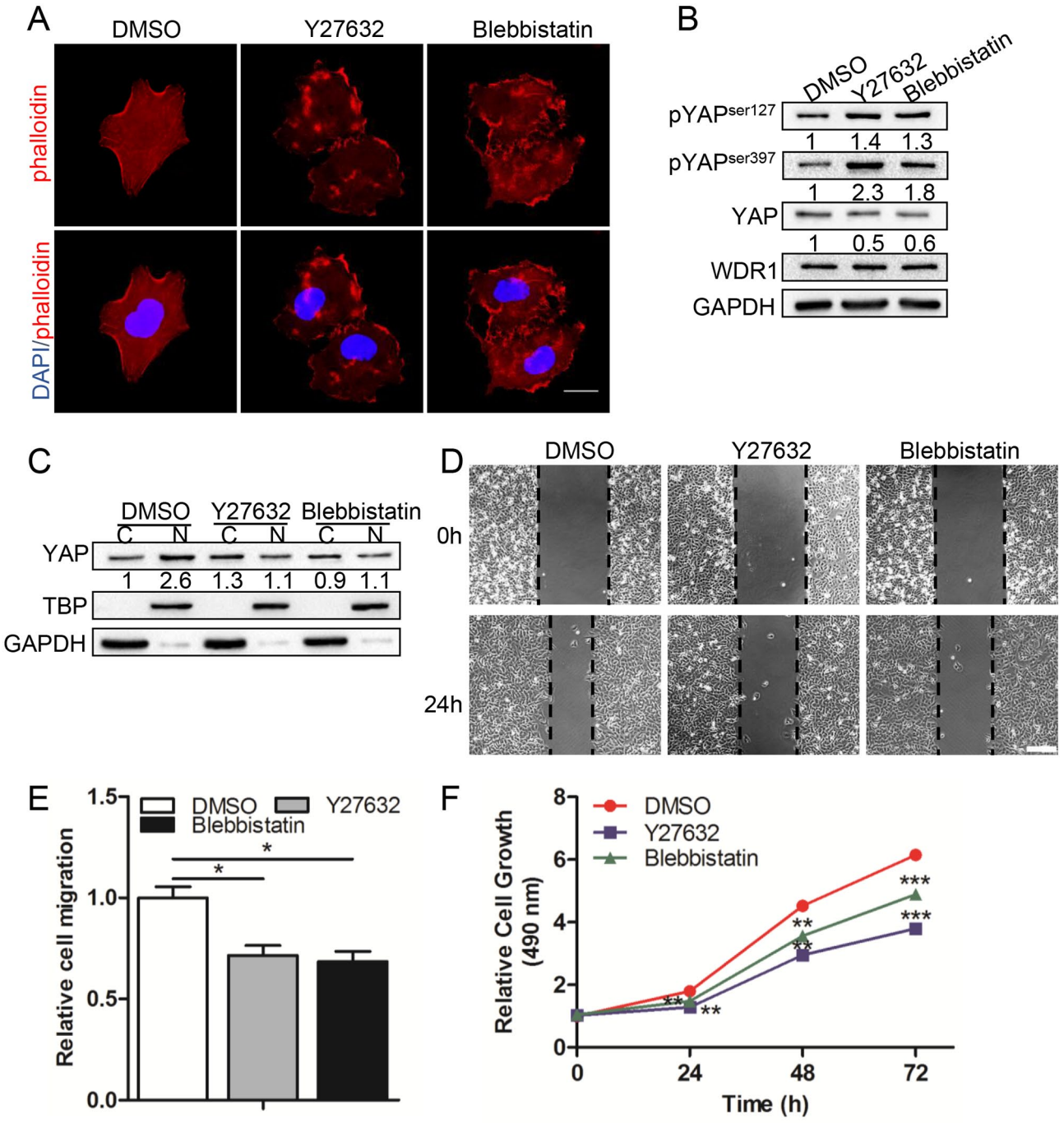

Figure 3. Disruption of cortical tension inhibited the signaling of YAP. (a) Immunofluorescence staining for phalloidin. Y27632 and blebbistatin treatments showed disrupted stress fibers compared to the control group, and F-actin showed an uneven and patchy distribution. Scale bar = 20 μm. (b) Western blotting analysis for indicated proteins. A549 cells treated with Y27632 and blebbistatin resulted in decreased expression of YAP but increased phosphorylated YAP. The quantified protein levels were shown beneath the corresponding bands. (c) Y27632 and blebbistatin treatments resulted in a significantly lower N/C ratio of YAP compared to the control group. (d) and (e) Scratch healing assays showed that Y27632 and blebbistatin treatments inhibited the migration of A549 cells. Scale bar = 200 μm. (f) CCK8 assay showed that the proliferation of A549 cells was significantly inhibited by treatment with Y27632 and blebbistatin. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

dramatically higher in the shWDR1 group (Figure 5(f)). In addition, LATS1/2 knockdown partially rescued the migration and proliferation in WDR1-knockdown cells, which indicated that YAP signaling contributed to WDR1-regulated migration and proliferation of NSCLC cells (Figure 5(g) to (j)).

We also confirmed that H226 cells (another NSCLC cell line) with stable knockdown of WDR1 showed significantly reduced YAP protein levels and the level of YAP phosphorylation was increased (Supplemental Figure S1(a)). Then, we performed LATS1/2 knockdown in shWDR1 cells. Western blot analysis revealed a significant decrease in the

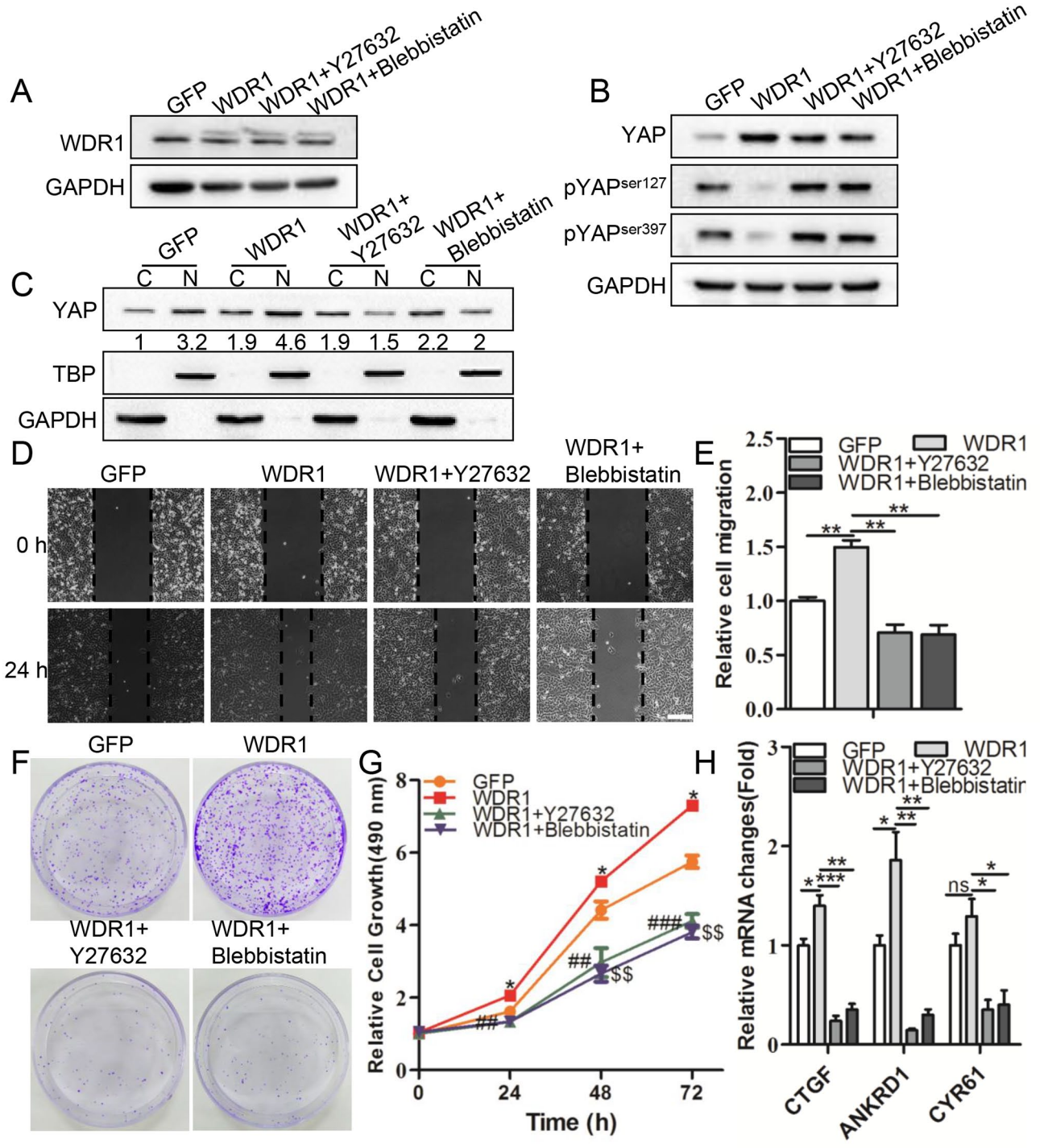

Figure 4. Y27632 and blebbistatin treatments disrupted the increased YAP signaling as well as the cell proliferation and migration in WDR1-over-expressed A549 cells. (a) Y27632 and blebbistatin did not affect WDR1 expression. (b) and (c) Y27632 and blebbistatin reduced phosphorylated YAP while partially reversing the increase in total YAP (b). Y27632 and blebbistatin partially reversed the increased N/C ratio of YAP (c). (d) and (e) The scratch healing experiment revealed that WDR1 promoted increased cell migration, and cell migration was significantly reduced after treatment with Y27632 and blebbistatin. Scale bar = 200 μm. (f) In WDR1-over-expressed cells, colony formation was greatly boosted, and the colony formation was dramatically inhibited after treatment with Y27632 and blebbistatin. (g) When compared to their corresponding controls, Y27632 and blebbistatin treatments dramatically reduced the proliferative rates induced by WDR1 over-expression. (h) Over-expression of WDR1 increased the expression of YAP/TAZ target genes. Y27632 and blebbistatin reversed the increased levels. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

level of phosphorylated YAP after LATS1/2 knockdown, and the total YAP also recovered compared to the shWDR1 group (Supplemental Figure S1(b)). In addition, LATS1/2 knockdown partially rescued the migration and proliferation in WDR1-knockdown cells (Supplemental Figure S1(c)). Consistent with previous data, these results further indicated that YAP signaling contributed to WDR1-regulated migration and proliferation of NSCLC cells.

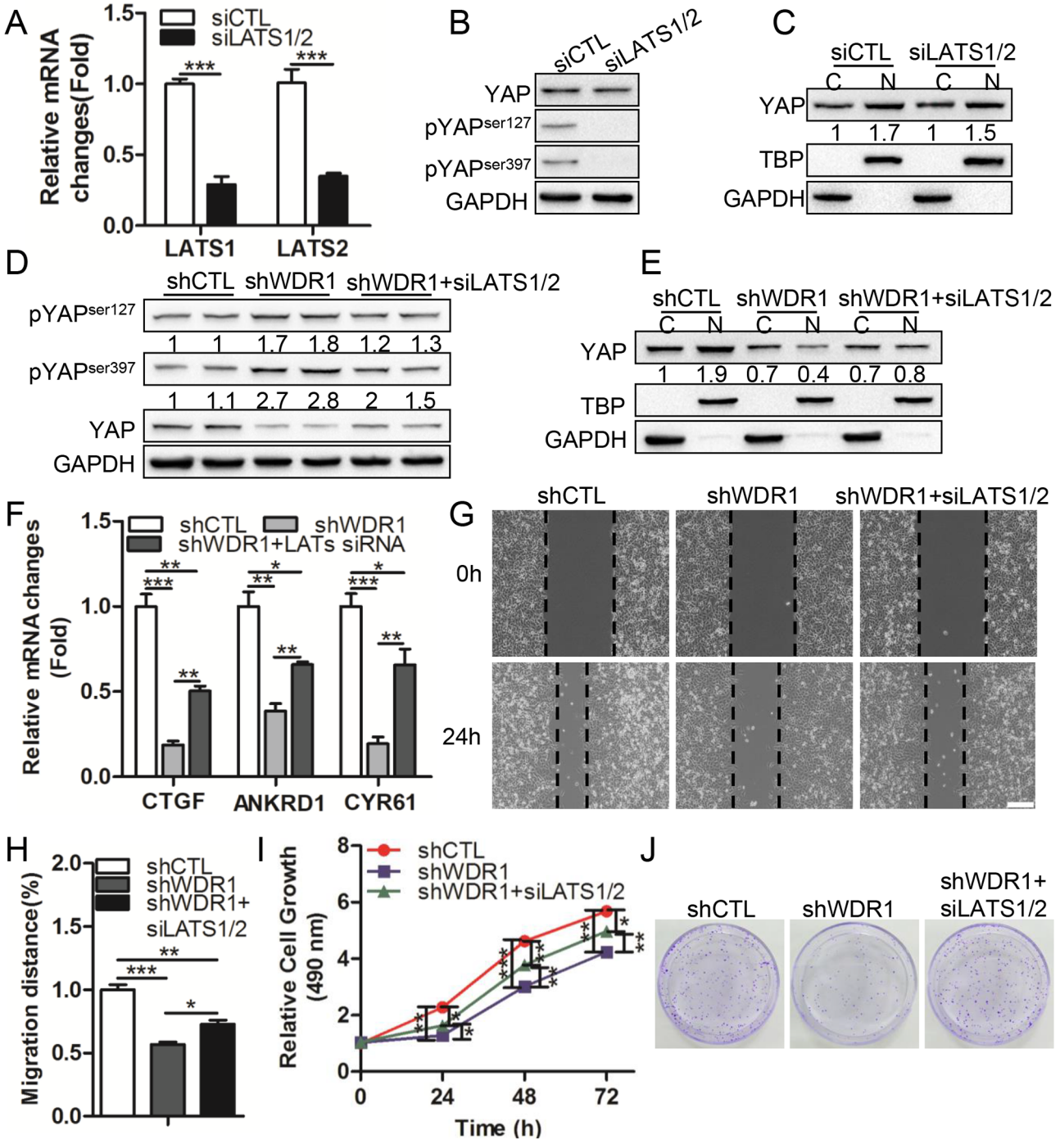

Figure 5. Hippo-dependent YAP signaling contributed to WDR1-regulated migration and proliferation. (a) The effectiveness of LATS1/2 knockdown was determined by RT-qPCR. (b) and (c) Western blotting for indicated proteins after LATS1/2 knockdown in A549 cells. Knockdown of LATS1/2 dramatically inhibited phosphorylation of YAP (b) and had no effect on the nucleo-cytoplasmic distribution of YAP (c). (d) and (e) LATS1/2 deficiency partially reversed the upregulated level of phosphorylated YAP and decreased level of total YAP. The quantified protein levels were shown beneath the corresponding bands. Nucleo-cytoplasmic separation results and western blot analysis showed that knockdown of LATS1/2 in shWDR1 cells increased the N/C ratio of YAP. (f) mRNA expression levels of YAP/TAZ downstream target genes. Knockdown of LATS1/2 in shWDR1 cells increased the expression of YAP/TAZ target genes compared to shWDR1 cells. (g) and (h) The reduction of LATS1/2 in shWDR1 cells increased cell migration in the scratch healing experiment. Scale bar = 200 μm. (i) CCK8 assay showed that knockdown of LATS1/2 partially rescued the decreased proliferation of shWDR1 cells. (j) Compared to shWDR1 cells, colony formation was remarkably increased in shWDR1 + siLATS1/2 cells. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

Nuclear exclusion is involved in inhibiting YAP signaling mediated by WDR1

To determine whether WDR1 knockdown reduced the Hippo pathway by entrapping YAP/TAZ in the cytoplasm or promoting their nuclear exclusion, leptomycin B (LMB), a nuclear export inhibitor, was used to investigate the effect of WDR1 on YAP nuclear exclusion. Western blot analysis indicated that treatment with LMB for 6 h had no significant effects

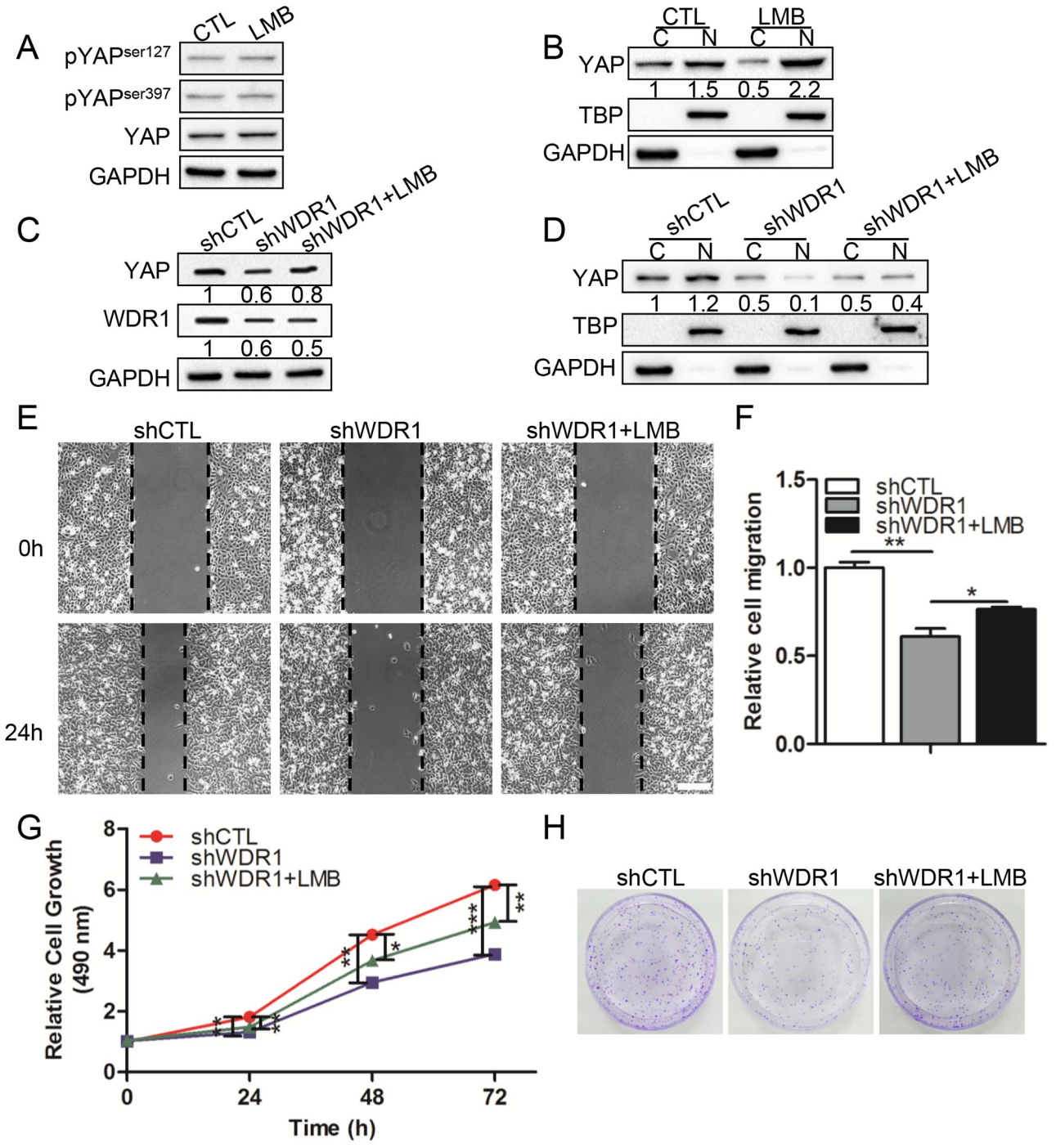

Figure 6. Nuclear exclusion is involved in inhibiting YAP signaling mediated by WDR1. (a) Western blotting was used to detect levels of total YAP and phosphorylated YAP after LMB treatment. (b) Nucleo-cytoplasmic separation assay. LMB therapy significantly enhanced the expression of nuclear YAP in LMB group, compared to the CTL group. (c) Western blotting for indicated proteins. LMB treatment increased the total YAP compared to the shWDR1 group. The quantified protein levels were shown beneath the corresponding bands. (d) The nucleo-cytoplasmic distribution of YAP was detected by western blotting after nucleo-cytoplasmic separation. Knockdown of WDR1, followed by LMB treatment, increased the N/C ratio of YAP. The quantified protein levels were shown beneath the corresponding bands. (e) Scratch healing assays showed that LMB treatment promoted cell migration in shWDR1 cells. Scale bar = 200 μm. (f) Quantification results of scratch healing assay. (g) CCK8 assay for A549 cells exposed to different treatments. In shWDR1 cells, LMB promoted cell proliferation. (h) The clonal formation assay showed the increased cloning ability in shWDR1 + LMB cells, relative to shWDR1 cells. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

on the total YAP and phosphorylated YAP (Figure 6(a)). By contrast, the nucleo-cytoplasmic separation results showed that LMB significantly increased the level of YAP in the nucleus compared to the control group (Figure 6(b)). After LMB treatment, the level of total YAP was increased in shWDR1 cells compared with the shWDR1 group (Figure 6(c)). Blockade of YAP nuclear export by LMB partially rescued the decreased nuclear translocation of YAP in WDR1-knockdown cells (Figure 6(d)). In parallel, we also analyzed the biological function of cells after LMB treatment. LMB treatment partially rescued the

inhibition of cell migration (Figure 6(e) and (f)) and cell proliferation in shWDR1 cells (Figure 6(g) and (h)). In summary, the reduction of nuclear YAP in shWDR1-treated cells could be attributed to the enhanced nuclear exclusion of YAP.

Discussion

Our investigation is the first one to reveal the mechanism that WDR1 regulates YAP signaling. Knockdown of WDR1 impaired the stress fibers and YAP signaling, and the disruption of cortical stress by drugs significantly inhibited YAP nuclear trafficking and enhanced YAP phosphorylation. In shWDR1 cells, knockdown of LATS1/2 reduced the increased level of phosphorylated YAP and rescued the level of phosphorylated YAP and cell migration and proliferation. Collectively, our data suggested that WDR1-mediated cortical stress might involve the regulation of Hippo-dependent YAP signaling.

YAP/TAZ are transcriptional co-activators which collaborate with other transcription factors, particularly TEAD family members, and recognize homologous *cis*-regulatory elements. It is reported that cellular tension regulates the activity of YAP,[32] and actomyosin-generated tension is critical for YAP subcellular distribution in heart proliferation capacity loss shortly after birth.[33] Furthermore, as mentioned above, WDR1 is required for cell ability to generate/maintain cortical tension.[7] Our data revealed that knockdown of WDR1 disrupts actin dynamics and stress fibers. Cells treated with blebbistatin and Y27632 showed consistent phenotypes to WDR1 knockdown, as stress fibers were disrupted. In WDR1-over-expression A549 cells, treatments with Y27632 and blebbistatin disrupted the increased N/C ratio of YAP, cell proliferation and migration, and endogenous target genes of YAP signaling. These results implied that WDR1-regulated YAP signaling might be dependent on actin cytoskeleton-mediated cortical tension. However, future experiments are needed to clarify the effect of WDR1-mediated cellular tension on YAP signaling, including the measurement of cellular tension. WDR1-knockdown promotes YAP nuclear exclusion, and LATS1/2 phosphorylates YAP. Phosphorylated YAP is retained in the cytoplasm and then degraded.[34] Blocking the Hippo/LATS signaling by siRNA LATS1/2 in WDR1-knockdown cells showed that LATS1/2 depletion partially rescued the decreased level of total YAP and increased phosphorylated YAP. Our data indicate that WDR1 regulates YAP signaling in a Hippo-dependent manner.

For malignancies caused by increased TEAD–YAP, there are currently no clinically approved targeted treatments.[35] TEAD–YAP interaction studies might lead to new treatment approaches for TEAD–YAP-activated malignancies.[36] Rho and actin cytoskeleton inhibition can inhibit YAP/TAZ transcriptional activity.[30] WDR1 is known as a co-factor of ADF/cofilin, and it plays a key role in the dynamic recombination of the actin cytoskeleton.[37] We further observed that knockdown of WDR1 impaired stress fibers in NSCLC cells; this might also explain why WDR1 controlled NSCLC cell migration and proliferation. ROCK inhibitors can inhibit YAP/TAZ transcription by disrupting the actin cytoskeleton.[38] Fascin-1 knockdown inhibited actin cytoskeleton rearrangement and decreased Hippo/YAP signaling in liver cancer cells.[39] Consistently, our data showed the same effect of WDR1 on regulating YAP signaling in NSCLC cells. Considering that YAP/TAZ are attractive therapeutic targets in cancers, our study highlighted that WDR1 and YAP might serve as novel biomarkers of NSCLC.[19,40] In addition, co-targeting actin dynamics and YAP may be a potent therapeutic strategy to manage NSCLC.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript; RA, JW, XC, RX, JH, ZL, and CW conducted the experiments, and RA, JH, and BY wrote the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

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