Cell adhesion to the extracellular matrix or to surrounding cells plays a key role in cell proliferation and differentiation and is critical for proper tissue homeostasis. An important pathway in adhesion-dependent cell proliferation is the Hippo signaling cascade, which is coregulated by the transcription factors Yes-associated protein 1 (YAP1) and transcriptional coactivator with PDZ-binding motif (TAZ). However, how cells integrate extracellular information at the molecular level to regulate YAP1’s nuclear localization is still puzzling. Herein, we investigated the role of β1 integrins in regulating this process. We found that β1 integrin-dependent cell adhesion is critical for supporting cell proliferation in mesenchymal cells both in vitro and in vivo. β1 integrin-dependent cell adhesion relied on the relocation of YAP1 to the nucleus after the down-regulation of its phosphorylated state mediated by large tumor suppressor gene 1 and 2 (LATS1/2). We also found that this phenotype relies on β1 integrin-dependent local activation of the small GTPase RAC1 at the plasma membrane to control the activity of P21 (RAC1)-activated kinase (PAK) of group 1. We further report that the regulatory protein merlin (neurofibromin 2, NF2) interacts with both YAP1 and LATS1/2 via its C-terminal moiety and FERM domain, respectively. PAK1-mediated merlin phosphorylation on Ser-518 reduced merlin’s interactions with both LATS1/2 and YAP1, resulting in YAP1 dephosphorylation and nuclear shuttling. Our results highlight RAC/PAK1 as major players in YAP1 regulation triggered by cell adhesion.

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The abbreviations used are: FAK, focal adhesion kinase; MEF, mouse embryonic fibroblast; qPCR, quantitative PCR; RFP, red fluorescent protein; ROI, region of interest; EdU, 5-ethyl-2’-deoxyuridine.

β1 integrin–dependent Rac/group I PAK signaling mediates YAP activation of Yes-associated protein 1 (YAP1) via NF2/merlin

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localization remains ill-defined and has never been directly investigated.

Initially, RHOA was identified as a critical regulator of YAP; however, more recently RAC1 and CDC42 were also found to be involved in its regulation (16–18). So far, how small RHOA family GTPases regulate YAP nuclear translocation remains elusive. Knowing that integrins are key regulators of this GTPase class, we wondered whether β1 integrins might regulate YAP by controlling RHOA GTPases.

Herein, we address the mechanistic role of β1 integrins in the regulation of YAP localization and thereby cell proliferation. We found that β1 integrin–dependent cell adhesion was critical for supporting cell proliferation in mesenchymal cells both in vivo and in vitro by controlling YAP signaling rather than MAPK cascade. Mechanistically, we showed that β1 integrins are required for localizing the GTPase RAC1 at plasma membrane extensions. There, RAC1 activates its effector PAK1 and initiates in a merlin-dependent manner the nuclear translocation of YAP. Indeed, we found that merlin binds LATS via its FERM N-terminal domain but also interacts with YAP with its C-terminal moiety. The interactions between merlin and YAP or LATS are down-regulated upon phosphorylation by PAK1 at Ser-518. Altogether our data revealed a novel signaling pathway orchestrated by β1 integrins to locally activate a RAC/PAK1 cascade and negatively regulate the inhibitory protein merlin.

Results

β1 integrins regulate mesenchymal cell proliferation in a MAPK-independent manner

To explore the function of β1 integrins in bone tissue, we inactivated the β1 integrin gene in osteoblasts using Osterix-driven Cre recombinase expression. Mice with an osteoblast-specific β1-integrin deletion survived to adulthood but suffered from a growth deficit along with a significant decrease in the absolute number of osteoblasts (Fig. 1A). Because β1 integrins are known to regulate cell proliferation, we wondered whether the reduced osteoblast numbers observed could be due to a reduced proliferative capability of those cells. Although TUNEL staining did not reveal any significant difference in apoptotic cell number (Fig. 1, B and C), a significant reduction in BrdU incorporation was observed in mutant animals (Fig. 1, D and E). Similarly, in vitro, the loss of β1 integrins in isolated osteoblasts resulted in a significant proliferation defect (Fig. 1F). To rule out any osteoblast-specific phenotypes, we isolated mouse embryonic fibroblasts (MEFs) and confirmed that the loss of β1 integrin expression was associated with a reduced proliferative capability of the cells (Fig. 1G). Although it was proposed that integrins are important regulators of ERK signaling (19), we could not rescue the proliferation defect of β1 integrin-deficient cells by activating the MAPK/ERK pathway (Fig. 1H). In addition, we did not detect any significant modification of ERK phosphorylation when β1 integrin-deficient cells were compared with wild-type cells (data not shown).

β1 integrins are required for YAP nuclear localization and cell proliferation

YAP-dependent gene expression has emerged as an important pathway regulating cell proliferation (20). Moreover, it was recently reported that YAP nuclear localization is controlled in a cell adhesion manner through integrins and SRC/FAK (21); therefore, we first asked whether the loss of β1 integrin expression was indeed associated with a defect in YAP nuclear localization, which might account for the reduced proliferation observed in β1-deficient cells.

When compared with wild-type cells that displayed a prominent YAP/TAZ nuclear localization, the lack of β1 integrins was correlated with a strong relocation of these proteins within the cytoplasm (Fig. 2, A and B). This observation was confirmed with the other clones analyzed (Fig. 2C), as well as in cell lines stably expressing a flag-tagged YAP (Fig. 2D). To further evaluate the involvement of the ERK/MAPK pathway in controlling YAP nuclear localization downstream of β1 integrins, we isolated MEF cells from mice bearing a constitutively active allele of K-Ras (K-RasG12D) (22) with one or two deleted alleles of the β1 integrin gene. Even in the presence of the activated allele of K-Ras, YAP was mainly cytoplasmic as soon as the β1 integrin chain was genetically ablated (Fig. 2E). Notably, YAP/TAZ nuclear localization was completely restored upon re-expression of the human β1 integrin subunit in β1-deficient mouse cells, showing a direct relationship between β1 expression and the nuclear localization of YAP/TAZ (Fig. 2, A and B). In line with these observations, biochemical fractionations revealed a decrease in the nuclear pool of YAP in β1-deficient cells (Fig. 2F). Along with the reduced level of nuclear YAP, the expression of its target genes was also down-regulated in β1-deficient cells (Fig. 3A). YAP was shown to be sequestered in the cytoplasm by 14-3-3 proteins after the activation of its upstream protein kinase LATS (7). In good agreement with this, we noticed that the phosphorylation of YAP on Ser-127 (required for 14-3-3 binding) increased upon the deletion of β1 integrins as well as the ratio of activated (phosphorylated) versus total form of LATS (Fig. 3B). Therefore, taken together, these data confirmed that β1 integrins are important players in controlling YAP nuclear localization most likely in a LATS-dependent manner.

Having shown that β1 integrins regulate nuclear localization of YAP, we wondered whether the proliferation defect that we observed upon removal of β1 integrins was the consequence of YAP nuclear translocation. Our data highlighted that LATS-dependent phosphorylation of YAP was up-regulated in β1-deficient cells. Therefore, we stably expressed in those cells a non-phosphorylatable form of YAP. The expression of YAPSSA in β1 integrin-null cells relocated YAP into the nucleus and up-regulated its target genes (Fig. 3, C and D). Importantly, the expression of YAPSSA fully restored β1−/− cell proliferation capabilities as quantified by BrdU incorporation (Fig. 3E). Therefore, our data highlighted the important role of YAP signaling in the control of cell proliferation downstream of β1 integrins.

Finally, to confirm this view, we performed unbiased transcriptomic analyses on wild-type and β1 integrin-deficient cells under optimal growth conditions. With the defined filtering and statistical criterion, 800 probes representing 555 well-annotated genes were identified as being differentially expressed between the two considered groups (supplemental Fig. S1). Known YAP/TEAD target genes were significantly down-regulated in β1−/− cells. Among those were ankrd1, ctgf, and cyr61
These results confirmed our previous RT-qPCR analyses (Fig. 3, A and D). Consistent with the above-mentioned proliferation defect, a number of cell-cycle regulators were also deregulated (supplemental Fig. S1). To directly estimate whether the YAP–TEAD complex might impact the expression of those important cell-cycle regulators, we analyzed p19Arf, p21CIP, and cyclin D2 expression by RT-qPCR in cell expressing YAP5SA. In contrast to what was measured in /H92521-deficient cells, the expression of YAP5SA in wild-type cells was able to up-regulate cyclin D2 and down-regulate p19Arf but not p21CIP when compared with control cells (Fig. 3E). This strongly suggests that some important cell-cycle regulators are transcriptionally modified by the YAP–TEAD complex.

**RAC1, but not CDC42 nor RHOA, controls YAP nuclear localization downstream of β1 integrins**

Although accumulating evidence pinpointed a role for FAK and SRC in integrin–dependent control of the Hippo pathway (21), little is known about the downstream effectors. However, members of the Rho GTPase family were shown to be involved in the control of YAP activity (10, 23, 24). It is well known that integrins also play a critical role in

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**Figure 1. β1 integrins regulate osteoblast cell proliferation in vivo and in vitro.** A, histomorphometric analysis of osteoblast number on wild-type (β1+/+) and Osx-Cre; β1+/+(β1−/−) 30-day-old mouse tibias. Graphs show the mean ± S.D. from five independent experiments. B, quantification of apoptotic (TUNEL-positive) and proliferating (BrdU-positive) cells in periosteum and trabecular bone in wild-type and mutant 30-day-old mouse tibias. n = 50. Statistical significance of differences was assessed by a two-tailed unpaired Student’s t test for three independent experiments. C, representative TUNEL staining. D, BrdU staining on trabecular bone sections from wild-type and mutant mouse tibias (hc, hypertrophic cartilage; tb, trabecular bone; bm, bone marrow). Scale bar, 40 μm. E, images of BrdU staining of trabecular bone sections. F, BrdU-based quantification of the proliferation rate of β1+/+ , β1−/− , primary mouse embryonic fibroblasts. (statistical significance of differences assessed by a two-tailed unpaired Student’s t test). G, in vitro proliferation rate of wild-type (β1+/+) and β1 integrin-deficient (β1−/−) osteoblasts. n = 50. Statistical significance of differences was assessed by a two-tailed unpaired Student’s t test for three independent experiments. H, BrdU-based quantification of the proliferation rate of β1+/+ and β1−/− osteoblasts or the β1−/− osteoblasts expressing human β1 integrin (rescue), constitutively active MEK (MEKQ56P), or nuclear-active ERK fusion mutant (MEK/ERKLA). Statistical significance of differences was assessed by a two-tailed unpaired Student’s t test for three independent experiments.
Figure 2. β1 integrins regulate YAP nuclear localization. A, immunostaining of YAP (red) and β1 integrins (9EG7, green) on β1f/f, β1−/−, and β1-rescued (β1resc) osteoblasts. Scale bar, 10 µm. B, statistical analysis of YAP nuclear to cytoplasmic ratio, n > 50 cells for each condition. β1f/f and β1−/− osteoblasts were spread overnight on fibronectin (10 µg/ml). Data are represented in a logarithmic scale. n = 50; statistical significance of differences was assessed by a two-tailed unpaired Student’s t test, and the box plot is representative of three independent experiments. C, immunolocalization of YAP in β1f/f and β1−/− osteoblasts (independent second clone no. 4.6). D, immunolocalization of FLAG-YAP (red) in β1f/f and β1−/− osteoblasts using anti-FLAG antibody. Nuclei were stained with DAPI. E, immunolocalization of YAP (red) and phosphotyrosine (PY, 4G10) (green) in β1f/f, K-RasG12D/−, β1−/−, and K-RasG12D/− MEFs. Nuclei were stained with DAPI. F, Western blot analysis of YAP phosphorylation. YAPpSer127 and total YAP in β1f/f and β1−/− osteoblasts after cell fractionation of the nuclear fraction (N) and cytoplasmic/membrane fraction (CM). Lamin B and tubulin were used as nuclear and cytoplasmic markers, respectively.
Figure 3. β1 integrins control proliferation via YAP transcriptional activity. A, RT-qPCR analysis of gene expression in β1−/− osteoblasts normalized to β1f/f cells (green line, set to 1). Results are from four independent experiments. Statistical significance of differences was assessed by a two-tailed unpaired Student’s t test. B, analysis of YAP and LATS phosphorylation (YAPpSer-127 and LATS1/2pSer-909), and total YAP and LATS in β1f/f, β1−/−, and β1resc (rescued) osteoblasts. Actin is shown as loading control. C, immunostaining of YAP on β1f/f and β1−/− osteoblasts expressing or not the YAP5SA mutant. Scale bar, 10 μm. D, RT-qPCR analysis of Cyr61 and CTGF mRNA in β1f/f and β1−/− osteoblasts and β1f/f and β1−/− osteoblasts expressing FLAG-YAP5SA. Statistical significance of differences was assessed by a two-tailed unpaired Student’s t test for three independent experiments. E, BrdU-based quantification of the proliferation rate of β1f/f and β1−/− osteoblasts expressing or not the YAP5SA mutant. n = 30. Statistical significance of differences was assessed by a two-tailed unpaired Student’s t test for three independent experiments. F, RT-qPCR analysis of cyclinD1 (CCND1), cyclinD2 (CCND2), p19ARF, and p21CIP (CDKN1A) mRNAs in β1−/− and β1f/f osteoblasts expressing FLAG-YAP5SA normalized to β1f/f osteoblasts (set to 1, green line). Statistical significance of differences was assessed by a two-tailed unpaired Student’s t test for three independent experiments.
the activation or the coupling of Rho GTPases with their effectors. Therefore, we wondered whether YAP nuclear localization driven by β1 integrins might also be regulated by Rho GTPases and, if so, which one.

To address this question, we used β1-deficient osteoblasts that displayed a dramatic defect in YAP nuclear localization to generate stable cell lines expressing constitutively activated forms of RHOA, RAC1, and CDC42. Then, we analyzed which...
Rho GTPase was able to induce the relocation of YAP within the nucleus. Surprisingly, activated RHOA was unable to restore the nuclear localization of YAP (Fig. 4, A and B). In line with this observation, we neither observed any significant difference in RHOA-GTP levels in wild-type versus β1-deficient cells nor any change in its cellular distribution (Fig. 4, C and D). In sharp contrast, RACG12V expression (and to a lower extent CDC42G12V) was associated with a significant increase in YAP nuclear localization in β1−/− cells (Fig. 4, A and E). Importantly, RACG12V expression restored YAP nuclear localization in β1-deficient cells but not their spreading defect (Fig. 4F). This strongly suggested that RAC1 and CDC42 might act downstream of β1 integrins in the signaling pathway that regulates YAP nuclear localization.

Because the expression of constitutively activated CDC42 and RAC1 could activate common effectors such as PAK, we wondered whether both small GTPase proteins were physiologically involved in this regulation. Hence, to discriminate between RAC1 and CDC42, we specifically inhibited their activities and analyzed YAP subcellular localization. The expression of a dominant-negative form of CDC42 (CDC42ΔN-17) did not result in any significant YAP redistribution in wild-type cells (Fig. 5A and supplemental Fig. S3). In sharp contrast, the inhibition of RAC1 activity either pharmacologically with ETH1864 or by the expression of a dominant-negative form (RACΔN-17) led to a significant YAP redistribution to the cytoplasm (Fig. 5A and supplemental Fig. S3).

As our previous data pointed out for a major role of RAC1, we next analyzed whether the expression of constitutively active RAC1 could reverse the proliferation defect observed in β1-deficient cells. Indeed, RACG12V expression in β1-deficient cells restored proliferation up to the level of control cells (Fig. 5B). This suggested that both RAC1 and YAP were in a common pathway to regulate β1-dependent cell proliferation. Indeed, the expression of RACG12V in β1-deficient cells induced a decrease in YAP phosphorylation to a comparable level to the one observed in β1+/+ cells (Fig. 5C). From these results, we concluded that RAC1 is involved in YAP signaling upon β1-dependent cell adhesion. Surprisingly, quantification of RACGTP levels in β1+/+ and β1−/− in whole-cell lysates did not reveal any significant difference (Fig. 5D). This discrepancy suggested that an altered RAC1 localization, rather than a global defect in its activity per se, resulted in YAP mislocalization observed in β1-deficient cells. Supporting this hypothesis, it was reported that cell adhesion regulates RAC1 plasma membrane localization (25), although the role of β1 integrin in this process was not addressed. To address this question directly, we performed immunostaining to analyze endogenous RAC1 localization in β1+/+ and β1−/− cells. As expected, in wild-type cells RAC1 frequently accumulated at protrusive cell edges; however, this localization was strongly reduced upon β1 removal (Fig. 5, E and F). To orchestrate actin dynamics, it was previously shown that RAC1 recruits cortactin at cell lamellipodia (26). Further supporting the defect in RAC1 membrane localization in β1-deficient cells, we observed that cortactin localization at cell edges was also significantly reduced in mutant cells (Fig. 5, G and H).

**PAK1, a merlin inhibitor, acts downstream of RAC1**

On our search of how RAC1 could affect YAP nuclear localization, we focused our attention on its effectors, the PAK family. Indeed, PAK1/2 are activated at the plasma membrane by RAC1 and/or CDC42, where they regulate membrane dynamics (27, 28). First, we asked whether impaired RAC1 targeting to the plasma membrane was translated into a defect in PAK1 activity. As expected, this activity, monitored by its phosphorylation, was reduced in β1-deficient cells but restored upon the re-expression of β1 integrins (Fig. 6A). Consistent with the defect in RAC1 recruitment to the plasma membrane, PAK membrane localization was reduced in β1-deficient cells but restored upon the expression of the constitutively active RACG12V (Fig. 6B). Altogether, these results supported the view that β1 integrins by regulating RAC1 localization at the plasma membrane would promote the recruitment and activation of its downstream effectors such as PAK and cortactin.

Having shown that RAC1 was required for controlling YAP nuclear translocation downstream of β1 integrins, and that PAK1 activity was reduced in β1-deficient cells, we wondered whether YAP nuclear translocation was also dependent on PAK activity. We transiently expressed in both wild-type and β1-deficient cells the constitutively active mutant of PAK1 (PAK1T423E) and analyzed YAP subcellular localization. As observed previously with the expression of activated RAC1, the expression of constitutively active PAK1 also rescued the defective YAP nuclear localization in β1-deficient cells (Fig. 6C), whereas the pharmacological inhibition of group I PAK using IPA3 (a specific inhibitor of this class) in wild-type cells, significantly reduced it (Fig. 6D). Similarly, the expression of a dominant-negative form of PAK1 (PAK1K299R) resulted in the delocalization of YAP out of the nucleus (Fig. 6E). Altogether, our data strongly suggest that β1 integrins control YAP nuclear localization in a RAC/PAK1-dependent manner.

**SRC acts downstream of β1 integrin but upstream of RAC/PAK1**

As mentioned previously, SRC was described to mediate YAP nuclear translocation downstream of integrins. SRC was also shown to be required for RAC1 activity (29, 30). Therefore, we wondered whether the loss of β1 integrin expression also impaired SRC activity. Indeed, we observed that in β1−/− cells SRC was not properly activated (Fig. 7A). Consistent with this observation, the expression of a constitutively activated SRC rescued β1-deficient cell proliferation as well as YAP nuclear accumulation (Fig. 7, B–D) and PAK1 activation (Fig. 7E). Yet, the inhibition of RAC1 was still able to block YAP nuclear translocation (Fig. 7D). Altogether, these data clearly indicated that SRC is downstream of β1 integrins and upstream of RAC1 in this signaling pathway.

**β1 integrins control YAP nuclear translocation through merlin**

Next, we hypothesized that merlin, a known regulator of LATS1/2 (31), might be regulated by PAK1, and this might be an important step in how β1 integrin mediated YAP nuclear translocation. Indeed, together with PKA, PAK was described to induce merlin inhibition by phosphorylation of the Ser-518
Cell adhesion control of YAP activation

A

B

C

D

E

F

G

H
Figure 6. Rac1 effector PAK1 acts downstream of β1 integrins to control adhesion-dependent YAP localization. A, Western blot analysis of PAK1 and PAK1 (K299R) in β1Δm, β1−/−, and β1−RES (rescue) osteoblasts. Actin was used as loading control. B, Western blot analysis of endogenous RAC1, GFP-RAC1 (GFP), and PAK1 in β1Δm, β1−/− and β1−/− stably expressing GFP-RAC1(G12V) after cell fractionation to isolate total cell membranes (M) and cytoplasm (C). Tubulin and RalA were used as cytoplasmic and membrane markers, respectively. C, right panel, immunolocalization of YAP in β1−/− osteoblasts transiently transfected with the constitutively active eGFP-PAK1 (PAK1 (K293E)). Scale bar, 10 μm. Left panel, statistical analysis of YAP nuclear to cytoplasm ratio in β1−/− and β1−/− transfected with activated PAK1. Cells were spread overnight on fibronectin (10 μg/ml), n > 20 cells, represented in a logarithmic scale; statistical significance of differences was assessed by a two-tailed unpaired Student’s t test, and the box plot is representative of two independent experiments. D, statistical analysis of YAP nuclear to cytoplasm ratio, represented in a logarithmic scale. β1Δm were spread on fibronectin (10 μg/ml) in the absence or presence of IPA3 (PAK1 inh) and then stained for YAP. n > 50 cells; statistical significance of differences assessed by a two-tailed unpaired Student’s t test, and the box plot is representative of two independent experiments. E, statistical analysis of YAP nuclear to cytoplasm ratio represented in a logarithmic scale. β1Δm were mock-transfected (Ctl) or transfected with the dominant-negative form of PAK1 (PAK1 (K299R)), and after 48 h cells were seeded on fibronectin (10 μg/ml) for 1 h and processed for PAK1 and YAP immunostaining. PAK1-positive cells were selected to quantify YAP nuclear to cytoplasm ratio. n > 30 cells; statistical significance of differences assessed by a two-tailed unpaired Student’s t test, and the box plot is representative of two independent experiments.

residue. Monitoring merlin phosphorylation by Western blotting revealed a decrease in Ser-518 phosphorylation in β1-deficient cells when compared with parental β1 Δm cells (Fig. 8A). Such modification should favor the recruitment and activation of LATS. Indeed, merlin phosphorylation at Ser-518 was proposed to stimulate the intramolecular FERM to C-terminal interaction (32). In its non-phosphorylated form, merlin adopts an open conformation in which the FERM domain interacts
with LATS, and the C-terminal interacts with AMOT (another important effector of YAP signaling).

To confirm this, we performed GFP-trap experiments with several merlin domains with or without mutations. As reported by others, LATS was shown to interact with the FERM domain of merlin but not with its C-terminal moiety. Moreover, this interaction was significantly reduced with the phospho-mimetic S158D mutant (Fig. 8B). The deletion of a stretch of 7 amino acids within the FERM domain (named “blue-box”) was reported to act as a dominant-negative form when expressed both in Drosophila and mammals. Of importance, LATS interaction with merlin was strongly reduced in the blue-box mutant as compared with the full-length or FERM domain of merlin (Fig. 8B). We took advantage of this mutant (named NF2BB in this study) and generated stable cell lines expressing either a wild-type or a blue-box-mutated form of merlin. Expression of the NF2BB mutant in β1−/−β1f/f-deficient cells restored YAP nuclear localization (Fig. 8, C and D) suggesting (i) that merlin was

**Figure 7.** SRC regulates YAP nuclear translocation upstream of RAC1 and downstream of β1 integrins. A, Western blot analysis of SRC activation upon serum stimulation. β1f/f and β1−/− osteoblasts were serum-starved overnight, and then serum (10%) was added to the cells. Phosphorylation of SRC and YAP as well as the total amount was analyzed by Western blotting. Actin was used as loading control. B, EdU-based quantification of the proliferation rate of β1f/f and β1−/− osteoblasts expressing or not (Ctl) the constitutively active form of SRC (SRCYF). n = 50; statistical significance of differences was assessed by a two-tailed unpaired Student’s t test for two independent experiments. C, immunostaining of YAP (red) in β1f/f and β1−/− osteoblasts stably expressing or not the constitutively active form of SRC (SRCYF). Most right panel, β1−/− osteoblasts expressing SRCYF were treated with ETH1864 for 3 h prior to YAP staining. Scale bar, 10 μm. D, statistical analysis of YAP nuclear to cytoplasm ratio, represented in a logarithmic scale. β1f/f, β1−/−, and β1−/− expressing a constitutively active form of SRC (SRCYF) without (Ctl) or with ETH1864 (Rac1 inh) treatment. n > 50 cells; statistical significance of differences was assessed by a two-tailed unpaired Student’s t test, and the box plot is representative of two independent experiments. E, Western blot analysis of PAK1 activation. β1f/f and β1−/− osteoblasts expressing or not the constitutively active form of SRC (SRCYF) were analyzed for phosphorylated PAK1 (p423, activated form) and total PAK1. Actin was used as loading control.
Figure 8. β1 integrin control of YAP nuclear translocation in a merlin-dependent manner. A, Western blot analysis of NF2 and NF2Ser-518 in β1-deficient and β1+/+ osteoblasts. B, Western blot analysis of GFP trap performed with whole-length, N-terminal FERM domain, and C-terminal domain as well as the Ser-518 mutant of merlin to analyze interaction with LATS. Note that the Ser-518 mutation decreases LATS interaction. C, immunostaining of YAP (red) in β1+/+ and β1+/− osteoblasts (Ctl) or stably expressing NF2BB mutant. Scale bar, 10 μm. D, statistical analysis of YAP nuclear to cytoplasm ratio, represented in a logarithmic scale. Statistical significance of differences was assessed by a two-tailed unpaired Student’s t test, and the box plot is representative of two independent experiments. E, Western blot analysis of YAP, YAPSer-127, and merlin/NF2 (NF2) in β1+/+, β1+/− expressing or not the NF2BB mutant. Actin was used as loading control. F, EdU based quantification of the proliferation rate of β1+/+ and β1+/− expressing NF2BB osteoblasts were spread on fibronectin (10 μg/ml) and then stained for YAP. Statistical significance of differences assessed by a two-tailed unpaired Student’s t test for two independent experiments. G, RT-qPCR analysis of gene expression in β1+/+ and β1+/− expressing NF2BB osteoblasts gene expression was normalized to β1+/+ cells (green line, set to 1). Mean ± S.D. value of four independent experiments. H, left panel, statistical analysis of YAP nuclear to cytoplasm ratio, represented in a logarithmic scale. β1+/+ and β1+/− expressing NF2BB were spread on fibronectin (10 μg/ml) in the absence or presence of the Rac1 inhibitor ETH1864 and then stained for YAP. n > 50 cells; statistical significance of differences was assessed by a two-tailed unpaired Student’s t test, and the box plot is representative of two independent experiments. Right panel, statistical analysis of YAP nuclear to cytoplasm ratio, represented in a logarithmic scale. β1+/+ and β1+/− expressing NF2BB were spread on fibronectin (10 μg/ml) in the absence or presence of IPA3, then stained for YAP. n > 50 cells; statistical significance of differences assessed by a two-tailed unpaired Student’s t test, and the box plot is representative of two independent experiments.
required for YAP nuclear translocation downstream of β1 integrins and (ii) that the recruitment of LATs by merlin was important in this regulation. Consistent with these results, expressing the NF2mutant in β1-deficient cells also reduced YAP phosphorylation (Fig. 8E) and restored cell proliferation (Fig. 8F) and YAP target genes (Fig. 8G). Altogether, our data strongly support the view that during cell spreading, β1 integrins mediated the activation of the RAC/PAK1 axis to phosphorylate and inactivate merlin. Consequently, inactivated merlin results in LATs release and thereby YAP dephosphorylation. Finally, if this assumption was correct, the inhibition of PAK1 activity should block YAP nuclear translocation in a merlin-and LATs-dependent manner. We analyzed YAP subcellular localization in IPA3-treated β1cells expressing or not the NF2mutant. As expected, the inhibition of PAK1 significantly reduced YAP nuclear localization in control cells, although it had no significant effect in NF2-expressing cells, in line with the view that plasma membrane extensions were important sites of YAP phosphorylation (Fig. 9B).

It is noteworthy that in wild-type cells, RAC1 colocalized together with YAP at the cell edges, whereas its colocalization was strongly reduced upon removal of β1 integrins (Fig. 9C). We wondered whether proteins recruited at cell edges in a RAC1-dependent manner could also colocalize with YAP. Cells stably expressing GFP-cortactin showed an extensive colocalization during cell spreading, showing that YAP is enriched in a protrusive membrane region and suggested a proximity between a RAC1-based signaling with YAP (Fig. 9D).

To further characterize such a complex, we mapped the interactions between merlin and the different partners involved in YAP regulation. As mentioned above, LATs interaction with merlin was previously reported on the N-terminal FERM domain, whereas the AMOT was mapped to the C-terminal part of merlin (33). Because YAP could interact with both LATs and AMOT, we wondered which part of merlin was required for its putative interaction with YAP. We used HEK293 cells to express either the full-length GFP-merlin, the GFP-merlin FERM domain, or GFP-merlin C-terminal moiety. YAP and LATs association with merlin was analyzed after GFP pull-down. In contrast to LATs that interacts with merlin via its FERM domain, YAP was coimmunoprecipitated with the C-terminal moiety of merlin or full-length merlin but not by the N-terminal FERM domain (Fig. 10A). Altogether, these results strongly suggested that merlin serves as a scaffolding protein to bring into close contact the protein kinase LATs with its substrate YAP. Indeed, the phosphorylation of merlin at Ser-518 favors the close conformation of merlin and reduced its interaction with YAP compared with wild-type merlin (Fig. 10A).

Because the loss of β1 integrin was associated with a reduced YAP phosphorylation at Ser-518, this could be translated into a differential interaction between YAP and merlin. Indeed, immunoprecipitating YAP in β1-deficient cells recovered a significantly greater amount of merlin from the membrane pool when compared with wild-type cells (Fig. 10B). Again, inhibition of PAK1 led to a similar observation (Fig. 10C), strongly suggesting that PAK1 activation downstream of β1 integrins was involved in the control of an inhibitory complex encompassing LATs, merlin, and YAP according to the model presented in Fig. 10D.

Discussion

β1 integrins control cell proliferation in a RAC1/PAK/YAP-dependent manner

Although pioneering works have shown that integrins orchestrate the recruitment of growth factors and clustering of their receptors at the plasma membrane (likely via cytoplasmic effectors such as FAK and SRC), a clear picture of how integrins are involved in the control of cell proliferation is still missing (34). Recently, integrins and cell-matrix adhesion were proposed to participate in the regulation of the Hippo signaling pathway via SRC and FAK (21). However, from these data it was not established what are the downstream effectors of integrin/FAK/SRC and how this could be molecularly translated into YAP activation. Herein, we provide a molecular basis of the integrin control YAP nuclear translocation, and we decipher the final stage of this regulation.

The loss of β1 integrins was associated with a defect in osteoblast proliferation both in vivo and in vitro. Our work also confirmed β1 integrins as the main cell-surface receptors by which these cells are capable of linking YAP nuclear translocation in response to cell adhesion. Our observations are in line with previous reports showing that β1 integrins control cell proliferation in other tissues (4). Although our main data were obtained with osteoblasts, we observed a similar behavior in MEFs suggesting that the signaling pathway described herein applies to other adherent cell types. Although we focused our work on non-transformed cells, we recently reported that both β1 integrins and YAP are overexpressed in primary bone tumors in which they have been identified as poor prognostic markers (35). Knowing the role of β1 integrins during tumor progression and their capability to sense the extracellular environment, we can envision that β1-dependent YAP nuclear translocation may play an important role in the tumorigenesis of solid tumors.

Although the loss of β1 integrins in osteoblasts was clearly associated with a strong defect in YAP signaling that was responsible for the reduced proliferation observed in mutant cells, an important question that remains to be solved is why β1 integrins are so critically involved in controlling YAP nuclear localization. Indeed, we and others (11) have highlighted a specific role of β1 integrins in YAP nuclear localization. This ques-
Figure 9. Cellular localization of YAP and interactors at the cell edges. A, immunostaining of YAP (red) and LATS (green, upper panel) or NF2/merlin (green, lower panel) on β1<sup>f/f</sup> osteoblasts. Scale bar, 10 μm. Quantitative analysis by intensity correlation was performed to visualize and quantify the colocalization. B, immunostaining of YAP (red) and YAP<sub>PSer-127</sub> (green) on β1<sup>f/f</sup> osteoblasts (upper panels) or β1<sup>−/−</sup> (lower panels). Scale bar, 10 μm. Quantitative analysis by intensity correlation was performed to visualize and quantify the colocalization between YAP and its phosphorylated form. C, immunostaining of YAP (green) and RAC1 (red) on β1<sup>f/f</sup> osteoblasts (upper panels) or β1<sup>−/−</sup> (lower panels). Scale bar, 10 μm. D, immunolocalization of YAP (green) and TagRFP-cortactin in membrane extensions in β1<sup>f/f</sup> osteoblasts spread on fibronectin (10 μg/ml). Scale bar, 10 μm.
tion is even more intriguing knowing that both β1 and β3 integrins can regulate FAK and SRC (36). Clearly, additional work focusing on these early signaling events will be required to address this question.

Actin cytoskeleton was proposed to be critical for controlling YAP nuclear translocation, but the identification of a clear mechanism was elusive. Actin cytoskeleton remodeling may modulate YAP activity through the Rho GTPases family (23, 24). Our present data support a critical role for RAC1 rather than RHOA or CDC42 in good agreement with recent reports showing that Arl4c triggers YAP nuclear translocation via the up-regulation of RAC1 while blocking RHOA activity (37). Although our data do not highlight any role for RHOA in YAP nuclear translocation downstream of β1-dependent cell adhesion, we cannot rule out that this small GTPase as an important regulator of actin networks in turn may control membrane targeting of important players of the Hippo pathway under specific conditions. It is noteworthy that during cell spreading, integrin engagement inhibits RHOA activity to dynamically regulate actin cytoskeleton re-organization (38). This decrease in RHOA activity corresponds in a timely manner to YAP nuclear translocation; therefore, a direct role of the latter GTPase in adhesion-dependent YAP nuclear translocation is very unlikely. Similarly, suspended cells display an elevated level of RHOA, and yet YAP is sequestered in the cytoplasm.

In the future, it will be important to gain a better insight into how those GTPases cross-talk in space and time regarding their activation and recruitment of their downstream effectors. The outcome of such a cross-talk may impact actin remodeling and YAP nuclear localization.

Here, we propose an integrated view of how β1 integrins regulate YAP nuclear translocation (Fig. 10D).Indeed, using
the expression of an activated form of RAC1 and PAK1 or a mutant of merlin (loss of function), we rescued the defective YAP localization that characterizes β1 deficient cells. In contrast, blocking RAC1 and PAK activity in control cells impaired YAP nuclear localization. Together these data, with the observation that RAC1 and its effector PAK1 did not accumulate at the plasma membrane in β1-deficient cells, strongly support a picture in which β1 integrins regulate RAC1 delivery to locally activate PAK1 that in turn modulates YAP in a merlin-dependent manner. Fitting with this view, merlin, a well-known PAK substrate, is underphosphorylated in β1-deficient cells, a post-translational modification that favors its active state. Activated merlin promotes its interaction with LATS and YAP thereby blocking YAP nuclear translocation.

β1 integrins regulate the formation of a YAP inhibitory complex

It appears that several key players such as NF2/merlin and LATS that negatively regulate YAP are also concentrated in plasma membrane extensions. This observation fits with previous data showing that LATS is recruited and activated by NF2/merlin at the cell membrane (31) and with the localization of NF2/merlin in membrane ruffles (39). Our data extend the picture and showed that β1 integrins and PAK1 negatively regulate YAP–merlin interactions at the plasma membrane. The reduced YAP–merlin interaction is likely due to the capacity of PAK1 to phosphorylate merlin at the Ser-518 residue to limit YAP and LATS access. Recently, it has been reported that merlin phosphorylation at Ser-518 reduces its interaction with AMOT family members (a YAP interacting partners) (33). However, AMOT recruitment to merlin induces and/or stabilizes merlin open conformation and in turn allows LATS binding on the FERM domain of merlin. Our data would favor such a model, in which merlin YAP and LATS belong to a membrane-associated inhibitory complex that may be dissociated upon PAK1 phosphorylation. Indeed, we observed that LATS and YAP interact with non-overlapping domains of merlin. Although LATS is recruited on merlin using its FERM domain, YAP interacts with the C-terminal part of merlin. Although our data did not establish whether the YAP–merlin interaction is direct or via AMOT, they clearly indicate that merlin acts as a scaffold to bring in close proximity LATS with YAP. The β1-dependent regulation of PAK1 increases merlin phosphorylation and thereby decreases YAP and LATS recruitment on merlin. Therefore, we proposed that YAP and LATS are recruited in an inhibitory complex at the plasma membrane orchestrated by merlin. Upon cell adhesion, RAC1 and PAK1 are locally activated and induce merlin phosphorylation to disrupt merlin, YAP, and LATS complex, a prerequisite for YAP nuclear translocation (Fig. 10D).

Although our data provide insights into how YAP is controlled by β1 integrins upon cell adhesion, the mechanism of RAC1 targeting to the plasma membrane by β1 engagement is still elusive albeit extensively described. Recently, FAK, PI3K, and SRC were shown to regulate YAP nuclear translocation (11), but at the molecular level how those proteins control YAP via LATS was not investigated. It is noteworthy that RAC1 activity is modulated by FAK (40) as well as by PI3K/SRC (41). Our data add to these findings, showing that actually SRC belongs to the β1 integrin-signaling pathways that controls YAP localization. Therefore, an open possibility is that FAK- and SRC-dependent regulation of YAP nuclear translocation also relies on a mechanism that converges on the release of YAP from merlin upon RAC/PAK1 activation. Our data highlighted that the loss of β1 integrins specifically affects RAC1 at the plasma membrane, and thus we could speculate that SRC/FAK could be important for RAC1 activation/localization and that β1 integrins would specifically regulate RAC1 coupling to its effector PAK. Indeed, similarly to RAC1, SRC and FAK were also shown to be activated on endosomes upon growth factor stimulation. Once activated, RAC1 is then translocated to the plasma membrane in a microtubule- and cell adhesion-dependent manner (42). Cell adhesion is then important to regulate microtubule targeting to the plasma membrane (43, 44).

Experimental procedures

Mouse genetics

Mouse strain with floxed alleles of β1 integrin (Itgb1tm1Ref) have been described previously (45) and were kindly provided by Dr. R. Fässler (Max Planck Institute, Martinsried, Germany). The Osx1-GFP:Cre deletor mouse was described previously (46) and was kindly provided by Dr. A. McMahon. Conditional knock-in mice bearing the G12D mutation at the K-Ras locus (KrasG12dtm1Def) were obtained from the NCI mouse repository and originally generated by Dr. T. Jacks (47). Mice were kept under regular conditions of husbandry accordingly to the European rules and approved by the University Ethical committee.

Cell lines and mesenchymal stem cell culture

Primary MEFs were isolated at embryonic day 14.5 (E14.5) from K-RasG12D β1+/f or K-RasG12D β1−/− embryos using a standard procedure. Cells were immortalized with the large SV40 T antigen. Immortalized K-RasG12D β1+/f and K-RasG12D β1−/− cells were infected with an adenoviral supernatant encoding the Cre recombinase for 1 h in PBS supplemented with 2% FCS and 1 mM MgCl₂. All other cell lines were generated upon retrovirus transduction, and transgene expression was verified by Western blotting and immunostaining.

Primary mesenchymal stem cells were isolated from WT and β1−/− bone marrow and selected on their capacity to adhere on plastic (48). The differentiation process was visualized by alkaline phosphatase staining described previously (49), and the number of alkaline phosphatase colonies having a diameter higher than 0.5 mm was evaluated using a stereomicroscope (Olympus SZX10).

Antibodies and expression vectors

Anti-BrdU, anti-FLAG (M2), anti-YAP/TAZ, anti-phospho-YAP, phospho-LATS, anti-LATS, anti-phospho-ERK, anti-ERK, and phospho-PAK were from Cell Signaling Technology (Ozyme, St Quentin en Yvelines France), and human β1 integrin 9E6G7 was produced from hybridoma. Anti-phospho-ELK, anti-YAP, αPAK, and RHOA were from Santa Cruz Biotechnology (Heidelberg, Germany). NF2...
and actin were from Sigma. β1 integrin clone 4B7R antibody from Abcam (Paris, France) was used. The anti-phosphotyrosine monoclonal antibody 4G10 used as hybridoma supernatant was produced in our laboratory. The human β1-expressing construct was based on the pCL-MFG retroviral vector as described previously (50). pBABEpuro-FLAG-YAP2 was from Dr. M. Sudol (Addgene catalog no. 27472). FLAG-tagged YAP2SSA from Dr. K. L. Guan (Addgene catalog no. 27371) was subcloned into the pQCXIP retroviral vector. pBABEpuro-MEK1T286D and pBABEpolyERK2MEK1-LA were kindly provided by Dr. M. Barbicad. pEGFP-RACG12V, pEGFP-RACG12V, pEGFP-RACG12V, pEGFP-RACG12V, and pEGFP-RACG12V were gifts from Dr. C. Gauthier-Rouvière, and pYFP-RHOA was from Dr. A. Mettouchi. The insert GFP-NF2BB were from Dr. T. Jacks (Addgene catalog nos. 14116 and 14117, respectively). GST-Rhotekin was from Dr. M. Schwartz (Addgene catalog no. 15247). IPA3 was from Sigma (l’Isle d’Abeau, France), and ETH1864 was from Tocris (R&D Systems, Lille, France).

**Transfections and infections**

HEK293 GP cells (Clontech, St Germain en Laye, France) were transfected with plasmid DNA using ExGen500 Transfection reagent (Euromedex, Souffelweyersheim, France) according to the manufacturer’s instructions. Osteoblast retroviral infections were performed as described previously (43).

**Histomorphometric analysis**

Tibiae were fixed and embedded in methyl methacrylate. Sections were deplasticized and stained for Masson-Goldner with hematoxylin (Gill II), acid fuchsin/Ponceau xylidine, and phosphomolybdic acid/orange G to stain the cells and osteoid and light green to stain the mineralized matrix (51). The total absolute number of osteoblasts in the area extending from 150 µm below the growth plate down 2 mm was evaluated and reported.

**TUNEL and BrdU in vivo staining assay**

Fluorescein “In Situ” Cell Death Detection Kit (Roche Diagnostics, Meylan, France) was used for TUNEL staining. Briefly, bone sections were deparaffinized and hydrated. Antigen retrieval and endogenous peroxidase quenching were performed and then TUNEL staining was achieved according to the manufacturer’s instructions. The TUNEL-positive cells and total cells (DAPI positive) in five areas of periosteum and trabecular bone from each of the mice in the experiments were counted under a ×20 objective microscope lens.

For BrdU staining, mice were sacrificed 2 h after being injected with BrdU (150 µg/g). Following deparaffinization and hydration, sections were treated 20 min with 4 N HCl, and then antigen retrieval was performed using trypsin 10 min at 37 °C. Finally, bone sections were immunostained for BrdU as described under “Immunofluorescence staining.”

**Immunofluorescence staining**

Cells were fixed with 4% paraformaldehyde/PBS for 15 min. Following permeabilization in PBS/Triton X-100 (0.2%) and blocking with goat serum (PBS/goat serum 10%), cells were incubated with primary antibodies during 1 h. Secondary antibodies were used conjugated with Alexa 488 and Alexa 555 from Jackson ImmunoResearch (Interchim, Montluçon, France). Samples were mounted using Mowiol 4-88 reagents (Sigma, l’Isle d’Abeau, France) supplemented or not with DAPI (Life Technologies, Inc., St Aubin, France) and were analyzed using Axiosimager microscope or LSM 510 laser-scanning confocal microscope (Carl Zeiss SAS, Le Pecq, France).

**Cell fractionation**

All the operations were carried out at 4 °C. Cells from four 10-cm Petri dishes were washed twice with PBS and then scraped into 1 ml of PBS with a rubber policeman. They were centrifuged at 1500 rpm for 5 min. The pellet was resuspended in a hypotonic buffer made of 10 mM HEPES, pH 7.4, 1 mM EDTA, and a mixture of protease inhibitors (Complete, Roche Diagnostics, Meylan, France) and phosphatase inhibitors (Sigma, l’Isle d’Abeau, France) and incubated for 30 min at 4 °C. Unbroken cells and nuclei were eliminated by centrifugation at 2500 rpm for 5 min. Mitochondria were further removed by a 6000 rpm centrifugation for 15 min.

For cytoplasm and total membrane recovery, the supernatant was centrifuged at 120,000 rpm at 4 °C for 20 min in a fixed angle AT120 rotor in a Hitachi micro-ultracentrifuge. The whole-membrane fraction was recovered from the pellet fraction after solubilizing in 50 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA for 20 min at 4 °C. Cytoplasmic proteins were recovered from the previous supernatant.

**RHOA and Rac1 activity**

GST-Rhotekin and GST-PAK-Crib based pulldown assays were carried out as reported previously (52).

**Pharmacological inhibition of Rac1 and group I PAK**

Cells were resuspended in DMEM and preincubated in suspension for 30 min at 37 °C. Then either ETH1864 (53) or IPA3 (54) was added at the concentration of 50 and 10 µM, respectively, and the incubation was pursued for another 30 min. The cells were then plated in the presence of the inhibitors for 1 h before fixation with paraformaldehyde and immunostaining.

**Immunoprecipitations**

GFP-Trap magnetic beads were used following the manufacturer’s instructions (Chromotek, Martinsried, Germany). Immunoprecipitation using the FLAG epitope was done with M2 antibody-coupled magnetic beads (Sigma, L’Isle d’Abeau France).

**Immunoblotting**

Cells were lysed using RIPA lysis buffer containing protease and phosphatase inhibitors. Cell lysates were centrifuged at 15,000 rpm for 30 min at 4 °C, and supernatants were used for immunoblotting using standard protocol.
RNA extraction, reverse transcription, qPCR, and transcriptomic analyses

Total RNA was isolated using TRizol reagent (Thermo Fisher Scientific, Waltham, MA) and RNeasy kit (Qiagen, Courtaboeuf, France) following the manufacturer’s instructions. Total RNA quantification was performed using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA was reverse-transcribed with the iScript Reverse Transcription Supermix (Bio-Rad). Real-time qPCR analysis was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) on Bio-Rad CFX96. The integrity of the extracted RNAs was assessed with the Bioanalyzer 2100 and the RNA6000 nano kit (Agilent Technologies Inc., Santa Clara, CA). An RNA integrity number greater or equal to 7.00 was achieved for all samples. No sign of DNA contamination was detected in any of the samples analyzed. The starting amount of total RNA used for the reactions was 400 ng per sample for all samples. The Illumina Total Prep RNA Amplification kit (Applied Biosystems/Ambion, Austin, TX) was used to generate biotinylated, amplified cRNA according to the manufacturer’s recommendations. Hybridization, staining, and detection of cRNAs on Illumina Mouse WG-6 version 2 Expression BeadChips were performed according to the manufacturer’s protocol. The Mouse WG-6 version 2.0 BeadChip profiles more than 45,200 transcripts derived from the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq) database (Build 36, Release 22), the Mouse Exonic Evidence-based Oligonucleotide (MEEBO) set, and from exemplar protein-coding sequences described in the RIKEN FANTOM2 database. The Illumina I-Scan system was used to scan all Expression BeadChips, according to Illumina recommendations.

Using the Gene Expression Module 1.9.0 of GenomeStudio version 2011.1 software (Illumina), the Quantile normalization method was applied to the primary probe data. Processed probe data were then filtered according to the following criteria: minimal signal intensity fold change of 1.50 across all samples and minimal probe signal intensity absolute change of 150 across all samples. Filtered data were then log2-transformed, and the expression values were compared between the b1−/− cells and wild-type β1wt samples using Omics Explorer 3.2 (Qiucore, Sweden). Genes were considered differentially expressed when their expression level satisfied two criteria: the adjusted p value (q value) was < 0.01 (which corresponded to an |R| > 0.96 ii) the absolute fold change between the mean expression value in the samples from mutant cells compared with that in controls was >1.5. Two-dimensional hierarchical clustering analysis was performed using Omics Explorer 3.2 software on normalized data (mean = 0, variance = 1) with the average linkage option.

Cell proliferation assay

Cells were treated with BrdU or alternatively with EdU (10 μM, Sigma) during 1 h or 30 min for osteoblasts and MEFs, respectively. For BrdU staining, cells were fixed with Carnoy’s fixative (75% methanol, 25% glacial acetic acid) 20 min at −20 °C and then denatured using 2 M HCl for 1 h at 37 °C. Cells were then immunostained for BrdU as described earlier. BrdU-positive cells were counted under Axioimager microscope (Carl Zeiss, Inc.). For EdU staining, manufacturer’s protocol was used, after an incubation of cells with EdU for 30 min.

Quantification of YAP nuclear localization

Cells were immunostained with an anti-YAP, and immunomicroscopy was carried out with a confocal laser-scanning microscope (Zeiss LSM510) equipped with a ×63 planapo oil immersion objective (n.a. 1.4) and a pinhole set to 1 Airy. On each cell image, a ROI was defined positioned either within the nuclei or in the cytoplasmic area next to the nuclei envelope. Because the thickness of the two ROI positions were likely identical, the average fluorescence intensity is likely proportional to YAP concentration and was estimated using ImageJ public software. Within the same cell, the ratio of both fluorescence intensities reflects YAP concentration ratio in both compartments. This ratio was represented under a logarithmic scale to have an identical range for positive and negative ratios. Measurements were performed with n ≥ 50, and statistical significance was estimated with Student’s t test. Boxplots were performed using R public software.

Colocalization microscopy

Confocal images were taken using LSM510 Zeiss microscope. Visualization and quantification of colocalized pixels were carried out using Wright cell imaging (15) facility plugins of ImageJ.

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