The mechano-sensitive response of β1 integrin promotes SRC-positive late endosome recycling and activation of Yes-associated protein

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Abstract.

Yes-associated protein (YAP) signaling has emerged as a crucial pathway in several normal and pathological processes. Although the main upstream effectors that regulate its activity have been extensively studied, the role of the endosomal system has been far less characterized. Here, we identified the late endosomal/lysosomal adaptor MAPK and mTOR activator (LAMTOR) complex as an important regulator of YAP signaling in a preosteoblast cell line. We found that p18/LAMTOR1-mediated peripheral positioning of late endosomes allows delivery of SRC proto-oncogene, non-receptor tyrosine kinase (SRC) to the plasma membrane and promotes activation of a SRC-dependent signaling cascade that controls YAP nuclear shuttling. Moreover, β1 integrin engagement and mechano-sensitive cues, such as external stiffness and related cell contractility, controlled LAMTOR targeting to the cell periphery and thereby late endosome recycling, and had a major impact on YAP signaling. Our findings identify the late endosome recycling pathway as a key mechanism that controls YAP activity and explains YAP mechano-sensitivity.

Introduction.

The integration of biological and mechanical signals coming from the surrounding cells and the extracellular matrix (ECM) is crucial for development, tissue homeostasis, and tumor progression. In adherent cells, external cues are involved in the control of numerous processes, including the adhesion-regulated formation of signaling platforms at the cell surface (1), endocytosis and trafficking of signaling receptors (2-4), and consequently the control of specific
transcription factors and chromatin remodelers that shuttle into the nucleus to modulate gene expression (5-7).

The nuclear shuttling of Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) is directly controlled through ECM compliance and composition and also through cell shape and confluence (8). In the nucleus, YAP and TAZ interact with TEAD family members to drive or modulate gene expression (6). In turn, the expression of specific genes affects cell behavior, such as proliferation/differentiation and migration, thus integrating external cues for cells to adapt to their extracellular environment (9). Over the last decade, the core signaling pathway leading to YAP and TAZ nuclear translocation has been extensively studied, giving important insights into the cell physiology (9-10). Integrin-dependent cell adhesion and particularly β1 integrins are crucial for YAP nuclear translocation (11-12). Indeed, β1 integrin-dependent cell adhesion, through the SRC family kinases, leads to Rac1 recruitment and activation at protrusive cell borders to stimulate a PAK1-dependent cascade resulting in merlin phosphorylation. Phosphorylated merlin releases YAP from a merlin/LATS/YAP inhibitory complex, allowing its nuclear translocation (12).

Vesicular trafficking is emerging as an important process involved in cell signaling. While receptor activation mostly takes place at the plasma membrane, their endocytosis and their sorting between recycling and degradation compartments also are important for their signaling output. Late endosomes (LE) subcellular positioning is crucial for controlling the cell anabolic/catabolic status (13-16). LE peripheral or perinuclear positioning depends on kinesin and dynein activities, respectively. In addition, the LAMTOR complex, a LE/lysosomal scaffolding protein complex, that integrates several pathways, such as the MAPK and mTORC1 signaling cascades (16-18),
participates in LE positioning by inhibiting the Arl8/BORC complex involved in LE peripheral targeting (19-20). At the cell periphery LEs target focal adhesions (FAs) and regulate their dynamics during cell migration, although the exact mechanism involved is still puzzling (21). However, the role of this vesicular compartment in the regulation of the signaling pathway that controls YAP activation has not been described yet.

Here, we found that β1 integrin-mediated cell adhesion and mechanical inputs regulate LE peripheral dispersion concomitantly with YAP activation. By knocking down p18/LAMTOR1, a major LAMTOR complex subunit, we demonstrated that LAMTOR recruitment to LEs is required for YAP nuclear shuttling and for LE targeting to FAs. Finally, we found that β1 integrins, through integrin-linked protein kinase (ILK), allows the organization of a functional microtubule network that transports SRC-positive LEs to the cell periphery, a crucial process in the regulation of YAP nuclear shuttling.
RESULTS

LE distribution is mechano-sensitive.

β1 integrins integrate mechano-dependent inputs from the ECM and control vesicular trafficking (3,22). These receptors also specifically regulate the nuclear translocation of YAP, a bona fide mechano-sensitive regulator of gene expression (8,11-12,23). Therefore, we asked whether LAMTOR-positive LE distribution and dynamics were mechano-sensitive and could act as a molecular link between integrins and YAP activation. First, we verified that YAP activation was mechano-sensitive in the pre-osteoblast cell line used for this study. YAP expression was mainly nuclear in cells cultured on fibronectin-coated polydimethylsiloxane (PDMS) medium and stiff hydrogels (10 and 30 kPa). Lowering compliance to 2 kPa led to a significant YAP redistribution in the cytoplasm (Figure 1A-B). Next, we analyzed LAMTOR complex distribution under the same experimental conditions, by monitoring the localization of p18/LAMTOR1 fused to GFP (p18-GFP), one of the main subunits of this complex. In cells grown under high stiffness conditions, p18-positive vesicles were both peri-nuclear and peripheral, whereas in cells grown in low stiffness conditions, the amount of peripheral p18-positive vesicles was significantly reduced (Figure 1C-D). We observed a similar effect of the matrix rigidity on LE distribution when GFP-Rab-7 was used as LE marker (Figure 1C). These data demonstrate that YAP mechano-sensitive response is correlated with LAMTOR-positive LE dispersion towards the plasma membrane. As β1 integrins are key mechano-receptors that controls YAP nuclear translocation (11-12,24), we asked whether β1 integrins also control LE subcellular distribution. After transduction of viral particles that express p18-GFP and RFP-VASP (to reveal FAs) in parental (β1^f/f) pre-osteoblasts (control) and in pre-osteoblasts that lack the β1 integrin subunit
(β1−/−) (25), we observed that p18/LAMTOR1-positive vesicles were at the cell periphery in control cells. Conversely, in β1−/− cells, the density of peripheral LEs as well as the number of LE targeting FA were significantly reduced (Figure 1E-F). This defect in LE distribution appears to be specific because when early endosome distribution was analyzed we did not notice any significant difference in their distribution between both genotypes (Figure 1G-H). Altogether, these data show that β1 integrins are crucial mechano-receptors controlling LE peripheral dispersion and YAP activation.

LAMTOR controls YAP nuclear translocation.

As the LAMTOR complex is implicated in the regulation of LE positioning in the cell (18,26), we investigated its role in YAP signaling. To this goal, pre-osteoblast cells expressing stable shRNAs against p18/LAMTOR1 (sh-p18 cells) were generated and characterized. It was reported that p18/LAMTOR1 is required for docking the LAMTOR complex to LE surface and consequently its loss leads to the complete inactivation of the complex (18). Indeed, the LAMTOR complex that was properly detected at LE surface in control cells or in rescue cells (sh-p18 expressing p18-GFP) was no longer observed upon p18/LAMTOR1 silencing (Figure S1A and S1B).

Having validated the functional loss of the LAMTOR complex upon p18/LAMTOR1 silencing, next we investigated whether this complex is involved in YAP signaling by analyzing its subcellular localization. With cells cultured on stiff substrate, the silencing of p18/LAMTOR1 significantly reduced YAP nuclear staining compared to control cells (sh-ctl; scramble shRNA) (Figure 2A-B). While not being specifically addressed in
this experimental setting, the re-expression of GFP-p18 restored the defective endogeneous YAP nuclear localization observed upon p18 silencing (Fig. 5G) indicating that the effect of p18 directed sh RNAs was not due to an off target silencing. In agreement with its reduced nuclear localization, YAP was reduced but hyper-phosphorylated in sh-p18 cells compared with sh-ctl and rescued cells (resc) (Figure 2C). The reduced level of YAP is likely reflecting its phosphorylation dependent degradation as previously reported (27).

As mentioned above the LAMTOR complex docks at LE surfaces using p18/LAMTOR1 and in particular its N-terminal moiety. Therefore, the addition of a tag to p18/LAMTOR1 N-terminus should interfere with its recruitment to the LE surface and this fusion protein should act as a dominant negative form. Therefore, to complement the silencing strategy, we generated a p18/LAMTOR1 chimeric protein in which the GFP protein was fused to its N terminus (GFP-p18). As expected for a dominant negative construction, GFP-p18 displayed a diffuse staining within the cells and the LAMTOR complex was no longer detected at the LE surface (Figure S1A and S1B). Further supporting the role of p18/LAMTOR1 in controlling YAP nuclear, those cells displayed a significant reduction in YAP nuclear localization (Figure 2D and Figure S1B). These results not only confirmed that LAMTOR mediates YAP nuclear localization, but also suggests that this process requires LAMTOR docking at the LE surface.

YAP signaling was reported to support osteoblast differentiation (8), and consequently sh-p18 cells is expected to present a defect in this process. Therefore, we investigated whether osteogenic differentiation was affected in sh-p18 pre-osteoblasts. Control and sh-p18 cells were cultured in osteogenic medium and alkaline
phosphatase activity (ALP) analyzed to visualize the initial stage of osteogenesis. At day 4 of differentiation, ALP staining was significantly reduced in sh-p18 cells compared with sh-ctl cells. At later differentiation stages (day 15), mineralization also was defective in sh-p18 cells, but not in control cells (Figure 2E). Altogether, these findings show that the LAMTOR complex has an important role in well characterized YAP signaling regulations.

**LAMTOR controls YAP nuclear translocation and LE targeting to FAs independently of the mTOR pathway**

Next, we aimed at understanding how the LAMTOR complex regulates YAP nuclear translocation. Since this later complex controls mTORC1 signaling (17,28), we investigated the potential role of mTORC1 in YAP signaling. Supporting previous findings, p18/LAMTOR1 silencing induced a decrease in the phosphorylation level of the S6 ribosomal protein, a well-known downstream target of mTORC1 (Figure 3A). Then, we tested whether the defect in YAP nuclear localization observed in sh-p18 cells was caused by downregulation of the mTOR pathway by inhibiting mTORC1 activity with everolimus. Upon incubation with everolimus, phosphorylated S6 was almost undetectable in β1/f/f pre-osteoblasts cultured on a stiff substrate, confirming the strong inhibitory effect of this molecule (Figure 3A). However, YAP subcellular localization was comparable in everolimus-treated and control cells (not treated), showing that mTORC1 activation is dispensable for YAP nuclear translocation on a stiff substrate (Figure 3B-C).

Having excluded the involvement of a LAMTOR-dependent mTOR signaling axis, we hypothesized that this complex might regulate YAP by controlling LE distribution.
In particular, it was reported that LEs are targeted to adhesive structures, such as FAs and fibrillar adhesions (21,29) from where they could initiate or modulate some signaling pathways. To further investigate this putative connection, we co-expressed GFP-Rab7 and RFP-paxillin (to label LEs and FAs, respectively) in both sh-ctl and sh-p18 cells. First, we confirmed that p18/LAMTOR1 silencing significantly affected LE subcellular positioning (Figure 3D-E). We also observed that LE vesicles (green in Figure 3E) were in close contact with FAs (red in Figure 3E) in control cells. Conversely, the number of vesicles targeted to FAs was significantly reduced in sh-p18 cells (Figures 3E and 3F for quantification). Altogether these data show that p18/LAMTOR1 is an important player in LE targeting to FAs. Importantly, LE positioning and targeting to FAs were not modified by incubation of β1(fo) pre-osteoblasts with everolimus, showing that mTORC1 is not involved in these processes (Figure 3G-I and movies 1 and 2). Collectively, these data indicated that LE dynamics and targeting to FAs are controlled by the LAMTOR complex in a mTORC1-independent manner.

**LAMTOR-positive LE subcellular distribution and targeting to FAs is β1 integrin/ILK- and microtubule-dependent.**

β1 integrin-mediated adhesion is crucial for YAP nuclear translocation (11-12,24), therefore p18/LAMTOR1 role in YAP nuclear translocation could be a consequence of a defective β1 integrin delivery to the plasma membrane and/or its recruitment to FAs. To monitor β1 integrin amount at the cell surface, we included Rab11-silenced cells (sh-Rab11) as positive control because Rab11 regulates β1 integrin recycling (30-32). First, we analyzed β1 integrin cell surface expression in sh-ctl, sh-Rab11 and...
sh-p18 cells by FACS. Compared with sh-ctl cells, the cell surface expression of β1 integrins was decreased in sh-Rab11 cells and increased in sh-p18 cells (Figure 4A). Quantification of the projected cell surface after 3 hours of spreading on fibronectin supported these findings. Indeed, Rab11 silencing promoted the partial rounding up of cells, but not p18/LAMTOR1 silencing (Figure 4B). Finally, immunostaining revealed that β1 integrins were localized in phosphorylated paxillin (pPaxillin)-positive FAs in both sh-ctl and sh-p18 cells, but not in sh-Rab11 cells (Figure 4C). These results strongly suggest that p18/LAMTOR1 controls YAP localization not by simply downregulating β1 integrin expression at the cell surface.

Together with the previous findings showing that β1 integrins regulate LE positioning, this suggested that β1 integrins are required for LE targeting to FAs. Indeed, the targeting of p18/LAMTOR–GFP-positive LEs to FAs was significantly decreased in β1-/- cells compared with control β1WT cells (Figures 4D and 4E). Similarly, dynamic analyses by total internal reflection fluorescence (TIRF)-based video microscopy demonstrated that p18/LAMTOR1 targeting to paxillin-labeled FAs was defective in β1-/- cells (movies 3 and 4). All these findings indicate that β1 integrin-dependent cell adhesion controls p18/LAMTOR1-positive LE trafficking and targeting to FA sites.

In addition to β1 integrins, ILK also has been implicated in YAP signaling (12,33). One of ILK functions is to scaffold proteins to allow microtubule anchoring to FAs (Figure S2A) (22,34). This suggests that the β1 integrin/ILK complex might be required for LE trafficking along the microtubule network. Indeed, we frequently observed p18/LAMTOR1-positive vesicles close to microtubules. Moreover, incubation with nocodazole (a microtubules inhibitor) led to
their relocation toward a perinuclear region (Figure S2A). In β1−/− cells, ILK expression at FAs was reduced and consequently, microtubule targeting to FAs as well (Figure S2B-D). Therefore, these data show that β1 integrin/ILK-positive FAs are required for microtubule anchoring that ultimately drives LE dynamics.

To more directly assess whether the microtubule targeting defect at FAs affected YAP nuclear translocation, we expressed a β3 integrin-ILK-GFP chimeric protein in β1−/− cells to force ILK localization to FAs. The chimeric protein was expressed at similar level than β3-GFP used as control (Figure S2E) and correctly incorporated in FAs, as previously reported (35). Microtubule targeting to FAs was restored in β3 integrin-ILK-GFP−, but not in β3 integrin-GFP-expressing cells (Figure 4F, upper panels). Immunostaining and quantification of the cytoplasmic/nuclear YAP ratio revealed a significant increase in YAP nuclear translocation in β3 integrin-ILK-GFP-expressing β1−/− cells when compared to β3-GFP-expressing β1−/+ or β1−/− cells (Figure 4F, lower panels, and Figure 4G). These data show that microtubule targeting to FAs is crucial for YAP nuclear translocation to control LAMTOR-positive LE targeting to FAs.

**p18/LAMTOR1 regulates SRC signaling.**

We next hypothesized that these LE vesicles could carry signaling proteins involved in YAP regulation. The non-receptor tyrosine kinase SRC, which is emerging as an important regulator of YAP nuclear shuttling upon integrin activation, is dynamically translocated from a vesicular pool towards the plasma membrane where it activates its downstream targets (36-37). First, to determine whether SRC could be detected on LE vesicles, we transiently co-expressed fluorescently tagged SRC (SRC-mCherry)
and p18/LAMTOR1 (p18-GFP) in pre-osteoblasts. As previously reported (37), confocal microscopy imaging revealed that the SRC-mCherry signal was not only diffused in the cytoplasm, but also appeared as punctuate staining. These vesicular-like structures were also p18/LAMTOR1-positive (Figure 5A). Quantification analysis revealed a Spearman coefficient >0.5 (significant) and thresholded Manders (tM1) values up to 0.7 when the green channel (p18/LAMTOR1) overlapped with the red channel (SRC-mCherry). The smaller tM2 value (red overlapping with green) might reflect a SRC fraction more broadly located to membranes (Figure 5B). Then, to assess whether SRC and p18/LAMTOR1 were dynamically coupled, we transiently transfected SRC-mCherry and p18-GFP in SRC, Yes and Fyn (SYF) triple knockout cells. Time lapse video microscopy confirmed SRC and p18/LAMTOR1 co-localization and co-trafficking in these cells (movie 5). Altogether these data showed that a pool of SRC is recruited to p18/LAMTOR1-positive vesicles. As we previously reported that p18/LAMTOR1 controls LE distribution, we asked whether it also regulates SRC distribution. Analysis of SRC distribution in sh-p18 and sh-ctl cells that express GFP-Rab7 (to visualize LEs) showed a significant co-localization of SRC-mCherry and GFP-Rab7 in control cells, further confirming SRC localization on LE. Conversely, in sh-p18 cells, SRC recruitment on LE was significantly reduced (Figure 5C-D).

Next, we investigated whether p18/LAMTOR1 silencing affects SRC activity by monitoring SRC phosphorylation at Y416 (pSRC\textsuperscript{Y416}) by Western blotting. In sh-p18 cells, total SRC and pSRC\textsuperscript{Y416} levels were strongly reduced compared with sh-ctl and resc cells (Figure 5E). This suggests that LE dynamics might regulate SRC sorting between recycling LEs (signaling) and the degradative endo-lysosomal compartment.
Immunofluorescence analysis did not allow detecting endogenous total SRC and \( \text{pSRC}^{Y416} \) on vesicles. Conversely, they were clearly present at FAs in sh-ctl cells, but barely visible in sh-p18 cells (arrows in Figure 5F, figure S3A). In sh-p18 cells, total SRC and \( \text{pSRC}^{Y416} \) signal in FAs was strongly increased upon expression of exogenous p18 (resc cells). Incubation with the mTORC1 inhibitor everolimus, which did not have any effect on LE distribution (Figure 3G), did not modify total SRC and \( \text{pSRC}^{Y416} \) signal at FAs (Figure 5F).

Next, we monitored the phosphorylation and localization of two well established SRC downstream target: \( \text{p130}^{\text{CAS}} \) and paxillin. In agreement with the reduced level of activated SRC in FAs of sh-p18 cells, \( \text{p130}^{\text{CAS}} \) phosphorylation also was reduced upon p18/LAMTOR1 silencing (Figure 5E). In sh-ctl cells, we observed \( \text{p130}^{\text{CAS}} \) phosphorylation mainly at FA sites, as expected, whereas it was markedly reduced in sh-p18 (Figure S3B). As previous studies showed that \( \text{p130}^{\text{CAS}} \) is phosphorylated at FAs upon stretching these data strongly suggest that p18/LAMTOR1 controls SRC delivery and activity at these sites (38). Conversely, paxillin phosphorylation was not modified in sh-p18 cells (Fig. 5E and figure S3C), suggesting that paxillin phosphorylation is not dependent on FA-associated SRC activity or alternatively involves another SRC family member such as Fyn or Yes. Together, these data highlight an important role for the LAMTOR complex in regulating SRC association with and expression in LEs.

Finally, to determine whether the defect in YAP nuclear translocation in sh-p18 cells was mediated by the lack of SRC activity, we transduced sh-p18 cells with the constitutive activated form of SRC (sh-p18+SRC\(^{YF}\)) and analyzed YAP subcellular localization (Figures 5G and H). Expression of activated SRC restored YAP nuclear
localization. We previously reported that SRC dependent regulation of YAP nuclear shuttling relies on the local activation of a Rac1/PAK1 axis that inhibits the formation of the inhibitory complex merlin/LATS/YAP (12). Accordingly, either the silencing of p18/LAMTOR1 or the pharmacological inhibition of SRC reduce Rac1 recruitment at cell edges (Figure S4). Altogether, these data showed that the LAMTOR complex is required for SRC localization on LE and its trafficking toward the plasma membrane, and specifically at FA sites, in order to drive YAP nuclear translocation likely via a Rac1/PAK1 axis.

Discussion.
In this study we identified the role of LE vesicular trafficking in the control of YAP activation. YAP is a well-known co-transcription factor that plays a key role in delivering information on the mechanical environments surrounding the cell to the nuclear transcription machinery. Along with TAZ, YAP is implicated in multiple cellular functions in tissue homeostasis and pathology. YAP and TAZ are both regulated by mechanical cues, and so far, little is known on their differential regulation. While being an interesting hypothesis, whether TAZ also depends on LE for its regulation is still an open question. Here, we demonstrated that β1 integrin-dependent cell adhesion allows the recycling of p18/LAMTOR1-positive LEs at adhesion sites by ILK-mediated anchoring of microtubules to FAs. This is required for the local delivery of the tyrosine kinase SRC at FAs, a necessary mechanism for YAP nuclear translocation (12,39). These findings identify p18/LAMTOR1-mediated LE recycling as an important player in YAP signaling regulation.

The LAMTOR complex controls LE-dependent delivery of SRC to FAs
This work adds to the growing evidence on the LE role as a major cell signaling compartment (17-18). It signaling function is tightly correlated to its dynamics/positioning and more specifically to LE capacity to recycle back to the plasma membrane near or at FAs (26,40). Indeed, LE localization (perinuclear versus peripheral) determines its function (catabolic versus anabolic) (13-14,16-17). Our present work supports this idea by showing that FA-associated LEs promote YAP nuclear translocation, a well-known cell growth promoter.

The LAMTOR complex, which was isolated from late endosomal detergent resistant membranes, is involved in the regulation of LE dynamics and signaling (16,18-19). In agreement, we observed that p18/LAMTOR1 has a critical role in LE targeting to the plasma membrane and FAs. Our data suggest that this is independent from its signaling function in the mTORC1 pathway. It was reported that LAMTOR restricts LE distribution to the perinuclear area by inhibiting the Arl8/BORC complex (19). We propose that LAMTOR is also required for the peripheral delivery of LEs that are targeted to adhesive structures. Although in apparent contradiction, these discrepant observations may be explained by the methods used to assess LE distribution and/or by the different cell types used. Indeed, in previous reports, LE distribution was analyzed by quantifying LE markers from the nuclear barycenter or the microtubule organizing center as the origin, without delimiting the cell borders. Here, we accurately delimitated the cell borders and quantified the vesicle densities from this position. This is particularly important in cells that generate large lamellipodia, such as mesenchymal cells. Indeed, we observed that upon p18/LAMTOR1 silencing, LE density was reduced mostly within the lamellipodial region.
One important LAMTOR function is to regulate mTORC1 activation. In agreement with previous reports, mTORC1 signaling appears to be dispensable for YAP nuclear translocation and also for LE positioning. Our present findings support the idea that, conversely, mTORC1 signaling is regulated by LE positioning and LATS1/2 (15-16,41).

One of our key findings is the identification of the LAMTOR complex role in the regulation of SRC local delivery to adhesion sites. We propose that SRC local delivery to FAs at the plasma membrane regulates signaling pathways involved in YAP nuclear translocation downstream of β1 integrin-mediated cell adhesion, as previously reported (11-12,39). The connection between LE and adhesive sites is known, but the functional consequences were poorly understood. It was previously reported that LE docking at FAs regulates their turn-over and β1 integrin endocytosis (21,29). Here, we unraveled another function of these vesicles. Indeed, their targeting to FAs regulates downstream integrin-dependent signaling, as exemplified here by YAP regulation downstream of SRC. Our data extend and support the emerging role of SRC in regulating YAP nuclear translocation (11-12,42). While SRC involvement in this pathway has been nicely documented, its exact role is still under debate and different alternative mechanisms have been proposed. Indeed, SRC was shown to directly interact and phosphorylate YAP but also its upstream kinase LATS1/2, thus promoting YAP nuclear translocation (43-44).

While we focused here on the role of LE trafficking in YAP nuclear translocation, it remains to clarify in the future whether this trafficking could also control YAP/SRC interaction and/or its phosphorylation. This is even more important knowing that SRC is a well-known activator of Rac1 that also is involved in YAP nuclear translocation (45). Several lines of evidence support this latter finding: i) v-SRC induced cell
transformation relies on the PAK1/Rac1 binding protein β-Pix/Cool 1 that promotes Rac1 recruitment at membrane ruffles (45-47) and ii) β-Pix/Cool 1 was identified in a high throughput screen as a regulator of YAP nuclear translocation (48). Importantly, merlin phosphorylation by the Rac1 effector PAK-1 inhibits its interaction with both LATS and YAP, thus promoting YAP dephosphorylation and nuclear translocation (12). Accordingly, SRC activity reduction by p18/LAMTOR1 silencing or by pharmacological inhibition affected Rac1 localization at protrusive borders (Figure S4). Although, SRC might regulate YAP nuclear translocation through different pathways, it is clear that its translocation to the plasma membrane at or near FA sites is of critical importance. Interestingly, the plasma membrane is emerging as an important compartment for YAP regulation (49-50). Therefore, this pathway appears to be an alternative mechanism to a direct role of SRC on YAP and LATS1/2. It will be important to investigate the relative contribution of these specific pathways in the control of YAP activity.

β1 integrin-dependent cell adhesion regulates LE subcellular positioning and FA targeting.

Firm cell adhesion to the ECM is required for the optimal occurrence of many cellular processes. For instance, loss of cell adhesion promotes lipid raft endocytosis, while cell adhesion favors their return to the surface. This tight connection between the adhesive system and membrane trafficking might play a major role in several signaling pathways, such as differentiation, proliferation and anoïkis (2-3,51). How exactly cell adhesion is involved is still puzzling, but our data fully support previous studies highlighting the role of microtubules. Part of the microtubule network is anchored at the cell periphery through β1 integrins and ILK.
(22,34). In line with these data, loss of the β1 integrin subunit resulted in a defect of ILK localization at FAs that correlates with a reduce microtubules anchoring to FAs. Moreover, forcing ILK localization into FAs not only restored microtubule anchoring, but also YAP nuclear shuttling. Interestingly, we also observed an increase in YAP nuclear localization upon forced expression of the β3 integrin subunit (although still being less efficient than the β3-ILK construct). This could be of importance under pathological situation when β3 containing integrins are overexpressed. This could be largely explained by the fact that ILK recruitment to FAs is only facilitated by β1 integrins under normal levels of expression (ILK presence in FAs is weaker but still detectable even in absence of β1 integrins), while overexpression of β3 should also lead to an increase in ILK localization at FAs and thereby promote microtubule targeting. The loss of integrins is associated with defective microtubule targeting to FAs that induces a defect in LE subcellular positioning and dynamics. This is in line with previous data showing that LEs move along the microtubular network (52). In addition, we rarely observed LEs associated with FAs in the absence of β1 integrins. As IQGAP1 interacts with ILK and LAMTOR (p14/LAMTOR2) (21), it is tempting to hypothesize that the β1 integrin/ILK axis regulates both LE trafficking and docking to FAs.

This relationship between integrin, YAP signaling, and the extracellular environment, provides a rational framework for solid tumor cell progression. Although it is broadly admitted that YAP signaling is regulated by cell contractility and substrate stiffness, the mechanical cues affecting the vesicular trafficking involved in this process have attracted less attention. For instance, caveolin is involved in the mechano-dependent response of YAP and also in
detergent-resistant membrane trafficking (53-54). Moreover, it might promote Rac1 withdrawal from the plasma membrane upon cell detachment or reduced matrix stiffness (3,51,55). Therefore, vesicular trafficking, by controlling Rac1 internalization or accumulation through LE recycling, appears as a powerful mechanism to control YAP nuclear shuttling and thereby, anchorage-dependent growth.

**Experimental procedures**

**Cell lines.** The generation and characterization of the β1<sup>fl</sup> and β1<sup>-/-</sup> pre-osteoblastic cell lines were presented previously (25). SYF fibroblasts, derived from Fyn, Yes and SRC triple knockout mice, were obtained from ATCC. From these original cell lines, the sh-p18, sh-Rab11a and sh-ctl cell lines were generated by transduction of lentivirus particles that express the shRNAs against p18 (Santa Cruz Biotechnology sc-36146-v), Rab11a (addgene # 26710, Dr K. Mostov) and scramble (addgene #17920, Dr S. Stewart). Cells were maintained in medium with puromycin. All other cell lines were generated by retroviral transduction, and transgene expression was verified by western blotting and/or immunostaining.

**Antibodies and expression vectors.** Anti-YAP<sub>S127</sub>, -Rab-11, -SRC, -SRC<sup>Y416</sup> -p130<sup>CASY410</sup>, -S6 and -pS6 antibodies were from Cell Signaling (Ozyme, St Quentin en Yvelines France). Anti-YAP, and -β-tubulin (clone 2.1) antibodies were from Santa Cruz (Heidelberg, Germany). Antibodies against mouse β1 integrin (MB1.2), mouse/human β1 integrin (9EG7), Rac1, and p130<sup>CAS</sup> were from BD Biosciences (Le Pont de Claix, France). The anti-actin antibody was from Sigma Aldrich (L’Isle d’Abeau, France). Anti-LAMP1 and -p-PAK antibodies were from Abcam. The anti-paxillinY31 antibody was from Invitrogen and the anti-paxillin antibody was from Millipore (Fontenay sous bois, France). The anti-phosphorylated
tyrosine (PY) monoclonal antibody 4G10 (hybridoma supernatant) was produced in our laboratory. Rabbit polyclonal antibodies against p18/LAMTOR1 were a generous gift by Dr. S. Manié (Lyon). The human β1-expressing construct was based on the pCL-MFG retroviral vector, as described previously (25). pCL-MFG-β3-GFP-ILK, pCL-MFG-β3-GFP and pCL-MFG-hILK-EGFP were a gift by Drs E. Van Obberghen and R. Fässler. pBABE-puro-FlagYAP2 was from Dr. M. Sudol (Addgene #27472). Flag tagged YAP2SSA was from Dr. K.L. Guan (Addgene #27371). The mCherry-YAP constructs were produced from these initial plasmids and cloned into pCL-MFG retroviral vectors. The pEGFP-Rac1G12V plasmid was a gift from Dr C. Gauthier-Rouvière. The GFP-Rac1G12V insert was subcloned into the retroviral vector pBaba-puro. Wild type and dominant negative mutants of Rab proteins were from Dr M. McCaffrey. Human wild-type paxillin cDNA in the pBABE vector was generously provided by Dr M. Hiraishi (Osaka Bioscience Institute, Osaka, Japan), and the mRFP-paxillin construct was generated from this initial vector. The pEGFP-N1-p18 construct was a gift from Dr Masado Okada (Osaka University, Japan) and was subcloned into the pCLM-FG retroviral vector. The pmCherryN1-SRC construct was from Dr S. Roche (CRBM, Montpellier, France). Everolimus and nocodazole were purchased from Sigma Aldrich.

Transfections and Infections. HEK GP 293 cells (Takara-Clontech, St Germain en Laye, France) were transfected with plasmid DNA using the TurboFect Transfection reagent (ThermoFisher scientific, Courtabeuf, France) according to the manufacturer’s instructions. Pre-osteoblasts were transduced with retroviral particles as previously described (56).

Immunohistochemistry. Cells grown on glass coverslips were fixed with 4%
paraformaldehyde (PFA) and 5% sucrose in PBS at room temperature (RT) for 10 minutes, and then permeabilized in 0.1% Triton X-100 in PBS for 5 minutes. Coverslips were washed twice with PBS, blocked in 1% BSA in PBS and incubated at RT with primary antibodies for 1 hour. Cells were rinsed in PBS and secondary antibodies were added at RT for 1 hour. Coverslips were mounted in Mowiol from Calbiochem (VWR International, Strasbourg, France) containing 4′6-diamidino-2-phenylindole (DAPI). Fixed cells were examined using a confocal laser-scanning microscope (LSM 510, Zeiss, Le Pecq, France), equipped with a Plan Apochromat 60X oil-immersion objective, N.A.1.4, with 2X zoom. The pinhole was adjusted to 1 Airy unit.

Quantification of YAP nuclear localization. Cells were immunostained with an anti YAP and images acquired with a confocal laser scanning microscope (Zeiss LSM510) equipped with a 63X plan-Apochromat oil immersion objective (n.a. 1.4) and a pinhole set to one Airy. On each cell image, a region of interest (ROI) was defined either within the nucleus, or in the cytoplasmic area next to the nuclear envelope. As the ROI thickness in the two positions was likely to be identical, the average fluorescence intensity should be proportional to YAP concentration in that area and was estimated using the Fiji public software. Within the same cell, the ratio of the fluorescence intensities in the nucleus versus the cytoplasmic area reflects the YAP concentration ratio in the two compartments. This ratio was represented with a logarithmic scale to have an identical range of positive and negative ratios. Measurements were performed with n ≥50 (unless otherwise indicated) and differences were compared with the Student’s t test. Boxplots were generated with the R public software.

Videomicroscopy and total internal reflection fluorescence (TIRF). For live
imaging, cells were seeded at sub-confluent densities on Labtech chambers and grown overnight in DMEM supplemented with 10% FCS prior to imaging on a 37°C heated stage in 5% CO₂ atmosphere (Carl Zeiss microimaging GmbH, Gottingen, Germany) with an Zeiss Axiovert 200M microscope equipped with a CoolSNAP HQ2, 100X (NA 1.4) Plan Apochromat objective and filters set to specifically detect Alexa488/GFP or Alexa546/pTRFP. Time lapse was 5 sec. TIRF microscopy was carried out with the same set-up equipped with the TIRF 1 slider (Carl Zeiss microimaging GmbH, Gottingen, Germany).

**Vesicle distribution.** Cell images were acquired with an Axioimager Z.1 microscope (Carl Zeiss Microimaging) equipped with a 63x/1.4 plan-Apochromat oil objective and an Axiocam Mrm CCD camera controlled by the Axiovision software. Images were then analyzed using the Icy software (http://icy.bioimageanalysis.org) with graphical programming tools for protocol editing. Channels were separated and processed to automatically detect cell borders with the best threshold, (HK-means and active contours plugins). In parallel, vesicles were detected using the wavelet spot detector block and the distance between the vesicle cell borders quantified using the ROI inclusion analysis plugin. The detailed protocol is provided upon request.

**PDMS hydrogels.** PDMS hydrogels of different stiffness (2, 10 and 30 kPa) were purchased from Excellness Biotech SA (Lausanne, Switzerland). Hydrogels were coated with bovine plasma fibronectin (2.5 µg/ml) according to the company’s protocol. Cells were seeded on such hydrogels and left at 37°C in CO₂ and humidified incubator for 2-3 hours. After washing, cells were fixed with 3% PFA/4% sucrose for 15 minutes, and then images were acquired as previously described.
**Colocalization analysis.** Images were obtained with a Zeiss Axiovert LSM510 biphoton confocal microscope equipped with a plan-Apochromat 63x/1.4 oil objective controlled by the LSM510 acquisition software. Optical sectioning was set to 0.8µm. Images were then analyzed using the Fiji software to run the JACOP plugin. Except when indicated, whole cell bodies were considered as ROI for analysis.

**Quantification of vesicle targeting.** Cells were imaged using an Axioimager Z.1 (Carl Zeiss Microimaging) microscope equipped with a 63x/1.4 plan-Apochromat oil objective and an Axiocam Mrm CCD camera controlled by the Axiosvision software. Images were then processed using the Fiji software. First, the background was subtracted using a rolling ball of 50 pixels. The p18-GFP and mRFP-VASP channels were merged and vesicles in direct contact with FAs were manually counted using the counter plug-in. Then, the mean value of the attached vesicles was divided by the number of FAs within the cell to obtain the vesicle/FA ratio. Twenty cells were analyzed for each experimental condition.

**Cell spreading.** Cells were spread on fibronectin-coated (5 µg/ml) Petri dishes for 3 hours, fixed in methanol, and stained with the Coomassie dye. Thresholded digital images were then processed using the Fiji software to quantify the cell area.

**FACS.** Cells were trypsinized, fixed in 3% PFA/PBS and β1 integrins detected by incubation with the MB1.2 antibody (1/100) at 4°C for 1 hour. β1<sup>−/−</sup> cells were the negative control.

**Statistics.** Cell analyses were carried out using at least 20 cells per experimental condition (for most of the presented experiments n ranged between 50 and 100). Statistical significance was estimated with the Student’s t test with bilateral distribution.
and unequal variance. A p value <0.01 was considered significant.

**Data availability.** All data are contained with the manuscript.
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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

Conceptualization: DB; Methodology: MRB, MB, MP and DB; Investigation: MRB, MB, TZ, GC, DB; Writing: MRB, MB, BWH, PR, CGR and DB; Supervision: DB and MRB.
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Legends to Figures.

Figure 1. Mechanical and β1 integrin-mediated regulation of YAP and LE subcellular localization in pre-osteoblasts.

A. Comparison of YAP cytoplasmic to nuclear ratios in different stiffness conditions. Pre-osteoblast cells that stably express mCherry-YAP\textsuperscript{wt} were seeded and grown on fibronectin-coated PDMS hydrogels of different stiffness (30 kPa, 10 kPa, 2kPa) for 2h. YAP subcellular localization was then analyzed by fluorescence imaging with a confocal microscope. Intensity values were obtained using the Fiji software (data are represented on a logarithmic scale). Data were compared with the two-tailed unpaired Student's \textit{t}-test, and are representative of 2 independent experiments with n>30 cells analyzed (* is p<0.01, ** is p<0.001 and *** is p<0.0001).

B. Subcellular localization of mCherry-YAP\textsuperscript{wt} (red) in pre-osteoblast cells spread on fibronectin-coated PDMS hydrogels of different stiffness (Young moduli are 30 kPa, 10 kPa, 2kPa) for 2h. Scale bar: 10µm.

C. Subcellular localization of p18/LAMTOR1 (upper panels) and Rab-7 (lower panels) in pre-osteoblast cells spread on fibronectin-coated PDMS hydrogels of different stiffness (Young moduli are 30 kPa, 10 kPa, 2kPa) for 2h. Scale bar: 10µm.

D. Comparison of p18/LAMTOR1-GFP subcellular distribution in pre-osteoblast cells spread on fibronectin-coated PDMS hydrogels of different stiffness (30 kPa, 10 kPa, 2 kPa) for 2h. GFP fluorescence was imaged and p18/LAMTOR1 distribution was analyzed using the Icy software. The histogram shows the localization of p18/LAMTOR1-positive vesicles from the cell edges to the nucleus (expressed as percentage of all vesicles). Data are the mean ± SD of 2 independent experiments. The localization of p18/LAMTOR1-positive vesicles was significantly
E. different only between the 20 kPa and 2 kPa conditions for 0-1 (*** and 1-2 (**) µm (two-tailed unpaired Student's t-test).

F. Control ($\beta_1^{\text{fl}}$) and $\beta_1$ integrin deficient pre-osteoblasts ($\beta_1^{-/-}$) that stably express p18/LAMTOR1-GFP (green) and mRFP-VASP (red) were grown overnight on glass coverslips and then imaged by fluorescence microscopy. Scale bar: 10µm.

G. Comparison of p18/LAMTOR1-GFP subcellular distribution in control ($\beta_1^{\text{fl}}$, green) and in $\beta_1$-deficient osteoblasts ($\beta_1^{-/-}$, blue) cells quantified using the Icy software. Histograms represent the localization from the cell edges to the cell nucleus and is expressed as percentage of all vesicles. The localization of p18/LAMTOR1-positive vesicles was significantly different only for 0-1 (*** and 1-2 (**) µm (two-tailed unpaired Student's t-test).

H. Control ($\beta_1^{\text{fl}}$) and $\beta_1$ integrin deficient pre-osteoblasts ($\beta_1^{-/-}$) were grown overnight on glass coverslips and stained for EEA1 to visualized early endosomes. Scale bar is 10 µm.

I. Comparison of the subcellular distribution of EEA1-positive vesicles in control ($\beta_1^{\text{fl}}$, green) and in $\beta_1$-deficient osteoblasts ($\beta_1^{-/-}$, blue) cells quantified using the Icy software. Histograms represent the localization from the cell edges to the cell nucleus and is expressed as percentage of all vesicles.

Figure 2. p18/LAMTOR1 is involved in YAP nuclear signaling.

A. Comparison of the YAP cytoplasmic to nuclear ratios (logarithmic scale) in pre-osteoblast cells that stably express mCherry-YAP$^{\text{wt}}$ and scramble (sh-ctl) or shRNAs against p18/LAMTOR1 (sh-p18) grown on glass coverslips overnight. YAP subcellular localization was analyzed by confocal microscopy and quantified with the Fiji software. Data are the mean ± SD of 2 independent experiments with n >30 (two-tailed unpaired Student's t-test).
B. Subcellular localization of mCherry-YAP\textsuperscript{wt} (red) in pre-osteoblast cells that stably express scramble (sh-ctl) or sh-RNAs against p18/LAMTOR1 (sh-p18). Scale bar: 10µm.

C. Western blot analysis of YAP, YAP\textsuperscript{pS127} and p18/LAMTOR1 expression in the indicated pre-osteoblast cell lines. Bands intensities were quantified with a Chemidoc CCD camera (Biorad) and ratios were calculated with the Image Lab software (Biorad). Actin was used as internal loading control. Representative of 3 independent experiments. Resc, sh-p18 cells that express exogenous p18/LAMTOR1-GFP.

D. Comparison of YAP cytoplasmic to nuclear ratios (logarithmic scale) in pre-osteoblast cells (β1\textsuperscript{ff'}) that express p18-GFP or GFP-p18 after overnight growth on glass coverslips. YAP subcellular localization was analyzed by indirect immunofluorescence and signal intensity was quantified from confocal images with the Fiji software. Data (mean ± SD) are representative of 2 independent experiments with n >30 cells analyzed (two-tailed unpaired Student's t-test).

E. In vitro osteogenic differentiation of pre-osteoblast cells (β1\textsuperscript{ff'}) that stably express scramble (sh-ctl) or shRNAs against p18/LAMTOR1 (sh-p18). Alkaline phosphatase (ALP) and Alizarin Red S (ARS; to detect calcium deposition) staining were performed to monitor differentiation status at day (D) 0, 4 and 15 as indicated. Scale bar: 5mm

Figure 3. p18/LAMTOR1 controls LE peripheral distribution and YAP signaling in a mTORC1-independent manner.

A. Western blot analysis of the ribosomal protein S6 (S6) and its phosphorylated form (pS6). Pre-osteoblasts (β1\textsuperscript{ff'}) were incubated (evero.) or not (ctl) with everolimus (10 nM, 3 hours). Bands intensity was quantified with a Chemidoc CCD camera (Biorad) and the Image Lab
software (Biorad). Actin was used as internal loading control. Representative of 3 independent experiments.

B. Comparison of YAP cytoplasmic to nuclear ratio (logarithmic scale) in pre-osteoblasts ($\beta^1_{1/f}$) grown overnight and then incubated (evero.) or not (ctl) with everolimus (10 nM, 3 hours) and stained for YAP. YAP subcellular localization was analyzed by confocal microscopy and intensity values obtained using the Fiji software. Data are the mean ± SD of 2 independent experiments with n >30 cells analyzed (two-tailed unpaired Student's $t$-test).

C. Immunostaining of YAP (green) in pre-osteoblasts ($\beta^1_{1/f}$) incubated (evero.) or not (ctl) with everolimus (10 nM, 3 hours). Cells were incubated with antibodies against YAP and phosphorylated tyrosines (PY) to label focal adhesions. Images were obtained with a confocal microscope. Bar: 10 µm.

D. Comparison of Rab7-GFP subcellular distribution in sh-ctl (green histograms) and sh-p18 (blue histograms) cells. Rab7-GFP distribution was analyzed using the Icy software. Data are the mean ±SD (2 experiments) of Rab-7 positive vesicle localization from the cell edges to the cell nucleus (percentage of all vesicles). Distribution was significantly different between conditions for (*) for 8 to 10; (**) for 10 to 12 and (***) for 0 to 3 (two-tailed unpaired Student's $t$-test). Due to the lack of appropriate tagged p18/LAMTOR1 construct, rescued cells were not investigated in this experiment.

E. Sh-ctl and sh-p18 cells that stably express mRFP-paxillin (red) were transiently transfected with GFP-Rab-7 (green). 24h post-transfection cells were seeded on glass coverslips and fixed overnight. Due to the lack of appropriate tagged p18/LAMTOR1 construct, rescued cells were not investigated. Scale bar: 10µm.

F. Quantification of GFP-Rab-7 targeting to focal adhesions in control (sh-ctl, red) and sh-p18 cells (green). Two-tailed unpaired Student's $t$-test, with n = 20 cells/condition.
G. Comparison of p18-GFP subcellular distribution in control (Ctl, red) and everolimus-treated cells (Evero., green). p18-GFP distribution was analyzed using the Icy software. Histogram represents the stepwise localization of p18-GFP-positive vesicles (percentage of all vesicles) from the cell edges to the cell nucleus, and data are the mean ±SD of three independent experiments (n >30 cells/condition; two-tailed unpaired Student's t-test).

H. Pre-osteoblast cells were incubated (Evero) or not (Ctl) with everolimus (10 nM, 3 hours) and p18-GFP distribution visualized. Cells were labeled with the anti-PY antibody to FAs. Bar: 10µm.

I. Quantification of p18-GFP targeting to FAs in control (red) and everolimus (10 nM, 3 hours)-treated cells. Data were compared with the two-tailed unpaired Student's t-test (n = 15 cells/conditions).

Figure 4. LE subcellular distribution and targeting to FAs are β1 integrin/ILK- and microtubule-dependent

A. FACS analysis of β1 integrin surface expression. Pre-osteoblast cells (β1^{Ef}) that stably express scramble (sh-ctl, green), or shRNAs against p18/LAMTOR1 (sh-p18, red) or Rab-11a (sh-Rab-11, blue) were stained with the monoclonal anti-mouse β1 integrin MB1.2 antibody.

B. Quantification of spreading of sh-ctl, sh-p18 and sh-Rab11 pre-osteoblast cells (β1^{Ef}). Cells were seeded and grown on fibronectin (5µg/ml) for 2h and cell spreading was quantified using Image J.

C. Immunostaining of β1 integrins (MB1.2, green) and phosphorylated paxillin (anti pY^{31}, red) of pre-osteoblast cells (β1^{Ef}) that stably express sh-ctl, sh-p18 or sh-Rab-11 spread overnight on glass coverslips. Due to the lack of appropriate tagged p18/LAMTOR1 construct, rescued cells were not investigated. Bar: 10 µm
D. Control (β1<sup>f/f</sup>) and β1<sup>−/−</sup> pre-osteoblasts that stably express p18/LAMTOR1-GFP (green) and mRFP-VASP (red) were seeded and grown overnight on glass coverslips and GFP and mRFP distribution visualized. Scale: 10µm.

E. Quantification of p18/LAMTOR1 targeting to FAs in control (β1<sup>f/f</sup>, green) sh-Rab-11 (red) and β1<sup>−/−</sup> pre-osteoblasts (blue). Data (mean ±SD of n >20 cells/condition) were compared with the two-tailed unpaired Student's t-test.

F. β1<sup>−/−</sup> (change in figure) pre-osteoblasts that stably express the β3-GFP (green, left) or β3-ILK-GFP (green, right) fusion proteins were seeded on glass coverslips and stained for microtubules (red, upper panels) or YAP (red, lower panels). Scale bar: 10µm.

G. Comparison of YAP cytoplasmic to nuclear ratio (logarithmic scale) in β1<sup>f/f</sup> and β1<sup>−/−</sup> pre-osteoblast that stably express or not the β3-GFP or β3-ILK-GFP fusion protein after overnight growth on glass coverslips. YAP subcellular localization was quantified from confocal microscopy images with the Fiji software; data (mean ± SD) from 2 independent experiments (n >30 cells/condition) (two-tailed unpaired Student's t-test).

Figure 5. p18/LAMTOR-dependent SRC delivery to the plasma membrane controls YAP nuclear shuttling.

A. Control pre-osteoblast cells were transiently transfected with p18-GFP and SRC-mCherry. 24h post-transfection, cells were seeded on glass coverslips and GFP and mCherry signals acquired by confocal microscopy. Scale bar: 10µm.

B. Histogram representing the Pearson coefficient and thresholded Manders (tM1, tM2) values obtained from confocal images of p18-GFP and SRC-mCherry co-transfected pre-
osteoblast cells. Images were analyzed using the Fiji Jacop plugin; data are the mean ±SD of >35 cells/condition (two-tailed unpaired Student's t-test).

C. Control (sh-ctl) and sh-p18 pre-osteoblast cells were transiently transfected with GFP-Rab-7 and SRC-mCherry. 24h post-transfection, cells were seeded on glass coverslips and the GFP and mCherry signals acquired by confocal microscopy. Due to the lack of appropriate tagged p18/LAMTOR1 construct, rescued cells were not investigated. Scale bar: 10µm.

D. Histogram representing the Pearson coefficient, and thresholded Manders (tM1, tM2) values obtained from confocal images of sh-ctl (red) and sh-p18 cells (blue) co-transfected with GFP-Rab-7 and mCherry-SRC. Images were analyzed using the Fiji Jacop plugin. Due to the lack of appropriate tagged p18/LAMTOR1 construct, rescued cells were not investigated. Data are the mean ±SD of >35 cells/condition (two-tailed unpaired Student's t-test).

E. Western blot analysis of SRC, phosphorylated (p) SRC\textsuperscript{Y146}, p130\textsuperscript{CAS}, pp130\textsuperscript{CAS}, paxillin, and ppaxillin\textsuperscript{Y311} form sh-ctl, sh-p18 and resc (sh-p18 with p18/LAMTOR1-GFP expression) cell lysates. Actin was used as loading control. Representative of 3 independent experiments.

F. Sh-ctl cells incubated or not (ctl) with (evero.) everolimus, sh-p18 cells, and sh-p18 cells that express p18-GFP (resc) were grown overnight on glass coverslips and stained for SRC and pSRC\textsuperscript{Y416}. Scale bar: 10µm

G. Comparison of YAP cytoplasmic to nuclear ratio (logarithmic scale) in sh-p18 cells, and resc cells (sh-p18 cells that express p18-GFP) that express or not the active form of SRC (SRC\textsuperscript{YF}) were seeded overnight on glass coverslips. YAP subcellular localization was quantified from confocal images using the Fiji software. Data are the mean ±SD of 2 independent experiments with >30 cells/condition (two-tailed unpaired Student's t-test).
Subcellular localization of YAP in sh-p18 pre-osteoblast cells and in sh-p18 cells that express constitutively active SRC\textsuperscript{YF} (sh-p18+SRC\textsuperscript{YF}). Scale bar: 10\(\mu\)m.
Fig. 1
**Fig. 2**

**Panel A**

Box plot showing the distribution of $\frac{YAP_n}{YAP_c}$ for sh-ctl and sh-p18.

**Panel B**

Immunostaining images of mCherry-YAP in sh-ctl and sh-p18 cells.

**Panel C**

Western blot analysis of p18, GFP, YAP, YAP$^{S127}$, ratio, and Actin. Molecular weights (kDa) are indicated.

**Panel D**

Box plot showing the distribution of $\frac{YAP_n}{YAP_c}$ for p18-GFP and GFP-p18.

**Panel E**

Images of ALP and ARS staining at D0, D4, and D15 for sh-ctl and sh-p18.
Fig. 3
Fig. 5
The mechano-sensitive response of β1 integrin promotes SRC-positive late endosome recycling and activation of Yes-associated protein
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