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The interaction between uPAR and VN triggers ligand-independent adhesion signalling by integrins

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1st Editorial Decision 28 January 2014

Thank you for the submission of your manuscript entitled "The interaction between uPAR and VN triggers ligand-independent adhesion signalling by integrins" to The EMBO Journal and please accept my apologies for the delay in responding due to the recent holiday break. Your study has been sent to three referees, and we have so far received reports from two of them, which I copy below. As both referees are convinced about the high interest, novelty and quality of your study, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this decision is made in the interest of time, and I will forward you the third report very likely including further requests, as soon as I receive it.

Without going into details that you will find below, both referees are very positive as I already mentioned. They express, however, besides a number of other important issues, rather fundamental concerns regarding the physiological relevance of your study that would need you attention during the review process. In particular, referee #1 considers that the use of a single cell line in vitro is not sufficient to sustain your conclusions. In line with this, referee #2 is concerned with the levels of proteins expressed in this cell line and, importantly, with the methods used to measure membrane tension.

Please be aware that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will essentially depend on the completeness of your responses included in the next version of the manuscript. Do not hesitate to contact me by e-mail or on the phone in case you have any questions, you need further input or you anticipate any problems during the revision process.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). Nevertheless, please contact me as soon as possible upon publication of any related work in order to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://emboj.msubmit.net/html/emboj_author_instructions.html#a2.12

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The manuscript by Ferraris and colleagues reports that binding of uPAR or an artificial PAI-1GPI receptor to vitronectin is sufficient to trigger b1 and b3 integrin outside-in signaling. The signaling event occurs only if cells adhere to a rigid surface, which is speculated to increase membrane tension. The authors demonstrate that uPAR/integrin ligand-independent signaling is indeed modulated by decreasing or increasing membrane tension, and requires the active conformation of integrins, the expression of talin and the integrity of the talin and kindlin binding sites in the integrin cytoplasmic domains.

This study is interesting, conceptually novel and well conducted. One drawback of this study is the lack of relevance as the entire study has been performed with an artificial in vitro cell culture system. It is possible that the mechanism described in the paper by Ferraris might be relevant for cells expressing low levels of integrins. In such a case they may play a minor role in attaching cells to the matrix proteins, while they are sufficient to trigger intracellular signals controlling, e.g. cell spreading. Another drawback is that the entire study is based on experiments with one cell line. It would be much more convincing if at least some experiments are repeated with another cells that e.g. lacks endogenous integrins and is reconstituted with the wild type or mutant integrin expression constructs.

The study is also provocative. An important unanswered question is how integrin, talin and kindlin become activated. The current dogma is that at least integrins and talins (for kindlin the mechanism has not been shown/discovered yet) have to undergo a conformational change to induce outside-in signaling. How is this achieved by uPAR? Additionally, the integrin-ligand independent outside in signaling described in this paper is independent on acto-myosin pulling forces exerted on integrins. Hence the integrin cluster should be small, and moreover, the GPI-anchored uPAR is responding to substrate stiffness. This all is interesting but hard to imagine how it can be achieved molecularly.

Comments:
(1) an integrin profile of the modified 293 cells would show the identity of integrins expressed on the cells.

(2) b1 integrins seem to be the predominant signaling receptors. Which b1 integrin mediates signaling?

(3) Western blot in Fig1 c shows a higher CAS phosphorylation in b1 integrin blocked cells. This does not fit.

(4) it is mentioned twice in the paper that integrins and uPAR do not undergo lateral interactions. There are no experimental data supporting this statement. This claim needs to be experimentally substantiated with convincing results.

(5) How do the adhesion sites of uPART54A cells on VN and VNRAD look in the presence and absence of uPA? Where do b1 and b3 integrins localize?

(6) Fig 2a and page 7: Cell adhesion of the uPART54A cells is approximately 2.5 fold weaker in the absence of uPA. Why?

(7) Talin and kindlin binding is required for integrin outside in signaling, most likely due to their role in recruiting and assembling the adhesome. Binding of talin and kindlin to integrin tails depends on inside-out signals triggered by numerous surface receptors. How are cell and the two adaptors "activated" by the GPI-linked uPAR or by the osmolality of the environment? Does an increase in membrane tension recruit the integrin regulators to the membrane followed by integrin activation, or does the increase in membrane tension result in the induction of an active integrin conformation followed by talin and kindlin binding?

(8) page 5 and figure 1: Figure 1e should be 1c as it is mentioned in the text after 1b and c.

(9) page 14: Fig8a and b should be Fig7a and b.

(10) Fig 7a: change labeling: top - not treated, bottom uPA-treated.

Referee #2:

The manuscript describes an interesting pathway by which uPAR-mediated adhesion to VN mediates integrin signaling through membrane tension and subsequent ligand-independent activation.

The manuscript is potentially very interesting as it describes a novel mechanism of receptor crosstalk through membrane tension. There are, however, two major concerns regarding the manuscript.

The first one is that all the major experiments are overexpression experiments, so it is not clear whether the mechanisms described occur with physiological levels of the receptors. What makes this particularly problematic is that the authors do not assess the cell surface levels of the expressed receptors let alone attempt to adjust the expression levels of the various mutants to comparable levels to exclude that the effects observed would be simply a consequence of differential degrees of overexpression. This type of adjustment is absolutely critical to allow conclusions and should be included for all receptors (uPAR, integrins and mutants thereof).

The second major concern deals with the assessment of membrane tension. The AFM measurements that are used to demonstrate higher membrane tension in uPAR expressing cells are inconclusive. The authors perform indentation experiments on the cell body and show that there is no difference in the membrane tension within the control and uPAR-expressing cell. They then proceed to measure the lamella of the uPAR-expressing cell and find it two have a higher membrane tension than the cell body. From this they conclude that uPAR expressing cells have higher membrane tension. This conclusion is unfounded as the control cells apparently do not have a proper lamella and this is not measured. It has been show that the density of actin highly contributes to the elastic modulus of a cell. As the lamella is a thin membrane structure with extremely high actin content, it is very likely to be stiffer than the cell body. Therefore comparing lamella of uPAR expressing cells to cell body of control cells to conclude that membrane tension is increased is not correct. The AFM measurements should be performed from lamella of both cell types. If the control cells really do not for any lamella, these types of measurements cannot be carried out.

Other points:

1) Does uPAR overexpression affect the cell surface levels of integrins? This should be addressed by FACS analysis.
2) Adhesion experiment are shown as percentage of cells adhering on a single time point. The question is whether these cells that do not adhere are incapable of adhesion all together (for example

because of not being viable) or whether adhesion is simply delayed. Adhesion time course experiments should be carried out, at least for the key experiments
3) In Western Blots for phospho-p130 Cas, total Cas should be also probed to exclude that overexpression or other manipulations affect total levels of p180Cas protein. The densitometric quantifications of phospho- Cas -levels should naturally

1st Revision - authors' response 28 April 2014

Referee #1:

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This study is interesting, conceptually novel and well conducted. One drawback of this study is the lack of relevance as the entire study has been performed with an artificial in vitro cell culture system. It is possible that the mechanism described in the paper by Ferraris might be relevant for cells expressing low levels of integrins. In such a case they may play a minor role in attaching cells to the matrix proteins, while they are sufficient to trigger intracellular signals controlling, e.g. cell spreading. Another drawback is that the entire study is based on experiments with one cell line. It would be much more convincing if at least some experiments are repeated with another cells that e.g. lacks endogenous integrins and is reconstituted with the wild type or mutant integrin expression constructs.

> The referee correctly points out that a drawback is that the study has been performed with an artificial *in vitro* cell culture system. We agree with the referee, however, the use of this cell system has been instrumental for us to identify, document and characterize ligand-independent integrin signaling and the unique requirement for plasma membrane tension. In cell lines where the predominant adhesion receptors are integrins, the disruption of ligand-engagement using mutations in the integrin, in the matrix ligand or applying inhibitory antibodies prevents cell adhesion and therefore also subsequent downstream cell spreading. We have in this study *uncoupled* cell adhesion from cell spreading using strong non-integrin adhesion receptors (uPAR and PAI-1gpi) as well as adhesion substrates promoting cell adhesion independently of integrin activation state (antibodies against uPAR, b_3 as well as poly-D-lysine). This uncoupling is critical to demonstrate that integrins transduce adhesion signaling even if they do not engage the ECM directly.

The referee points out that a drawback of the study is that it has been conducted with a single cell line. This is correct and to substantiate our findings we now present data documenting that ligand-independent integrin signaling is also observed in CHO cells overexpressing uPAR (new Sup. Fig. 2a) as well as in the MDA-MB-231 cell line expressing (patho)physiological uPAR levels (new Sup. Fig. 2b).

We fully acknowledge that we have not in this study determined the relative importance of canonical and ligand independent integrin signaling under physiological conditions. However, it has been documented elsewhere that ligand-independent activities of integrins are relevant in Drosophila development (Martin-Bermudo & Brown, 1999) and tumor progression (Desgrosellier et al, 2009). Furthermore, it is well described that uPAR plays important roles in tumor growth and that the direct interaction between uPAR and VN is at least partially responsible for this activity (Pirazzoli et al, 2013). We think these findings clearly underscore the importance and relevance of comprehensively understanding the signaling mechanism downstream of the uPAR/VN-interaction and other non-integrin adhesion receptors.

As our data show that the presence of active integrins is critical to transduce ligand-independent signaling downstream of uPAR, we do not expect this type of signaling to be active in cells expressing low levels of active integrins. In fact, our data show that two main requirements have to be met: firstly, the uPAR-expressing cells have to be exposed to a rigid VN-containing ECM and secondly, the cells have to express active integrins. Such conditions are for example met in

desmoplasia where activated stromal cells, often expressing high levels of uPAR, are located in a stiff fibrotic VN-containing tissue surrounding tumors.

The study is also provocative. An important unanswered question is how integrin, talin and kindlin become activated. The current dogma is that at least integrins and talins (for kindlin the mechanism has not been shown/discovered yet) have to undergo a conformational change to induce outside-in signaling. How is this achieved by uPAR? Additionally, the integrin-ligand independent outside in signaling described in this paper is independent on acto-myosin pulling forces exerted on integrins. Hence the integrin cluster should be small, and moreover, the GPI-anchored uPAR is responding to substrate stiffness. This all is interesting but hard to imagine how it can be achieved molecularly.

> The referee points out that an important unanswered question is how integrin, talin and kindlin become activated by uPAR. In this work we have not presented any direct evidence indicating that uPAR binding to VN *induces* integrin activation. We only document that an active conformation of integrins is *required* in the process. It is indeed fully possible that no integrin activation occurs and that uPAR simply utilizes integrins that are already in an active conformation. Extensive focused studies will be required to determine if and how uPAR binding to VN induces integrin and talin/kindlin activation.

We understand that the data illustrating the increased membrane tension in uPAR-induced lamellipodia and the increased integrin activation in cells under hypotonic conditions may, misleadingly, suggest a mechanism in which uPAR binding to VN induces membrane tension and that this increased membrane tension directly causes integrin activation. Although this is a possible mechanism, we do not have any data directly connecting uPAR binding to VN, induction of membrane tension and integrin activation. To avoid over interpretation of the data, we have therefore removed the data on integrin activation by hypotonic treatment (previous Fig. 7 panel d) as these are not directly pertinent to the main findings of this work. In response also to Referee #2, we have furthermore down-tuned our interpretation of the AFM experiments in the text and moved the data to a supplementary figure (new Sup. Fig. 5). We believe these changes make the manuscript more focused without any loss of significance.

Comments:

(1) an integrin profile of the modified 293 cells would show the identity of integrins expressed on the cells.

> We have previously reported the integrin profile of the 293 Flp-In cells utilized in this study (Madsen et al, 2007). The cells express the a_5b_1 receptor and this is responsible for cell adhesion to FN as confirmed also in this study by antibody inhibition experiments (Sup. Fig. 1d). The predominant VN adhesion receptor is a_vb_5 although the cells may also express very low levels also of b_3 ((Madsen et al, 2007) and this manuscript new Sup. Fig. 1e). We have not profiled the expression of other integrins as the above-mentioned receptors seem to be quantitatively responsible for the biological activities functionally investigated in this study. We have now complemented this analysis to cover also the 293/uPAR^{T54A} cells (+/- treatment with uPA) that are employed in the majority of the experiments of this study. The result of the analysis is that surface expression of b_1, a_5, a_vb_5 and b_3 are unaffected by the stimulus employed (new Sup. Fig. 1e) and we therefore conclude that possible differences in the surface expression of endogenous integrins are unlikely to condition the validity of the conclusions.

(2) b1 integrins seem to be the predominant signaling receptors. Which b1 integrin mediates signaling?

> We have in this study not formally demonstrated which is the b_1-heterodimer responsible for ligand-independent adhesion signaling as all the available evidences suggest a_5b_1. The a_5 subunit is highly expressed by 293 cells (new Sup. Fig. 1e) and the a_5b_1 heterodimer is responsible for cell adhesion to FN (Sup. Fig. 1d).

Also in response to point 5, we have now investigated the localization of b_1 by immunofluorescence and a_5 using a GFP-tagged version. The data are presented in the new figure 3 and supplementary movie 2. On VN and VN^{RAD} we observe a similar localization of b_1 and a_5 close to the leading edge of lamellipodia consistent both with the signaling heterodimer being a_5b_1 and the functional involvement in cell spreading.

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(3) Western blot in Fig1 c shows a higher CAS phosphorylation in b1 integrin blocked cells. This does not fit.
> In response also to Referee 2, other point 3, most of the signaling experiments have now been repeated using total p130Cas for normalization. The new blots are of better quality and also more accurately reflect the quantified data. Blocking b_1 partially inhibits p130Cas phosphorylation on VN^{RGD}, which fits.

(4) it is mentioned twice in the paper that integrins and uPAR do not undergo lateral interactions. There are no experimental data supporting this statement. This claim needs to be experimentally substantiated with convincing results.

> we seek not to over-interpret the data and, as pointed out by the referee, there are indeed no experimental data in the manuscript to document an absence of *direct* interactions between uPAR and b_1-integrins. What we believe our data do allow us to conclude is that possible direct interactions between uPAR and b_1-integrins are very unlikely to be *functionally* important in the signaling we have described here for two main reasons:

1) uPAR, PAI-1_{GPI}, and b_3^{Y2A} are structurally entirely different, yet do all three induce similar, if not identical, b_1-dependent signaling to cell spreading and p130Cas phosphorylation. This represents three out of three tested receptors and we find it exceedingly improbable that the biological activity of all three of these can be explained by specific direct protein-protein interactions with b_1. To us, it seems much more likely that the common activity of these three receptors is their ability to promote cell binding to the matrix as we argue in this work.

2) We have in the past shown that mutation of all the published integrin interaction sites in uPAR has no effect on the activity of the receptor to induce changes in cell morphology when seeded on VN (Madsen et al, 2007).

An interaction site for uPAR in b_1 has been published (Wei et al, 2005) and having here established a system for structure-function analysis of b_1, we have now also tested the activity of this variant in transducing ligand-independent signaling downstream of uPAR (data presented in Figure 4). Consistent with our finding using the PAI-1_{GPI} and b_3^{Y2A} adhesion receptors, as well as the previously published mutations in uPAR, this mutation fails to impair cell spreading.

To avoid any misinterpretations, we have in the revised manuscript furthermore replaced “direct interactions” with “functionally relevant interactions” to avoid any confusion. As substantiated above, we think this wording is justified by the data.

(5) How do the adhesion sites of uPART54A cells on VN and VNRAD look in the presence and absence of uPA? Where do b1 and b3 integrins localize?

> In response also to point 2, we have now included novel experimental data on the localization of b_1 and a_5 in cells spreading on VN and VN^{RAD} (new Figure 3, panel a and b as well as supplementary movie2). A prominent localization is observed close to the leading edge of lamellipodia consistent with the functional importance of b_1 in the uPAR-induced cell spreading on these substrates. There are furthermore no striking differences between VN and VN^{RAD} consistently with b_1 not directly engaging these substrates. Interestingly, the distribution is similar to that observed for unligated b_1 in the process of fibroblast cell spreading (Galbraith et al, 2007).

We cannot detect the very low levels of endogenous b_3 by immunofluorescence and the staining of cells with ectopic (over)expression of b_3 did not yield informative data. We have in the past extensively sought to follow the localization of b_1 and b_3 by time-lapse imaging using GFP-tagged versions of the receptors, however, for unknown reasons these display a very poor sorting to the cell surface and do not functionally rescue endogenous receptors in the 293 cells. With the localization data now presented in Figure 3 we nevertheless believe to have covered the more important receptor.

(6) Fig 2a and page 7: Cell adhesion of the uPART54A cells is approximately 2.5 fold weaker in the absence of uPA. Why?
> This is as it should be. The experimental advantage of this uPAR variant is that it has low baseline VN-binding that can be fully restored by addition of uPA. We have now explained this better in the

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text and have included new data on another uPAR-variant (uPAR^{W32A}), which documents that the phenotypic effect of treatment with uPA in 293/uPAR^{T54A} cells works exclusively through the induction of VN-binding. All the uPAR-variants have been extensively characterized in the past (Madsen et al, 2007).

(7) Talin and kindlin binding is required for integrin outside in signaling, most likely due to their role in recruiting and assembling the adhesome. Binding of talin and kindlin to integrin tails depends on inside-out signals triggered by numerous surface receptors. How are cell and the two adaptors "activated" by the GPI-linked uPAR or by the osmolality of the environment? Does an increase in membrane tension recruit the integrin regulators to the membrane followed by integrin activation, or does the increase in membrane tension result in the induction of an active integrin conformation followed by talin and kindlin binding?

> These are very good questions that we will certainly pursue in future experimentation as we believe that the work required to nail down the exact mechanism goes well beyond the current study. As previously explained, we actually do not even know if the integrin, talin and kindlin are activated in the process. We here only document that they are required.

(8) page 5 and figure 1: Figure 1e should be 1c as it is mentioned in the text after 1b and c.
> Corrected in the revised manuscript.

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Referee #2:

The manuscript describes an interesting pathway by which uPAR-mediated adhesion to VN mediates integrin signaling through membrane tension and subsequent ligand-independent activation.

The manuscript is potentially very interesting as it describes a novel mechanism of receptor crosstalk through membrane tension. There are, however, two major concerns regarding the manuscript.

The first one is that all the major experiments are overexpression experiments, so it is not clear whether the mechanisms described occur with physiological levels of the receptors. What makes this particularly problematic is that the authors do not assess the cell surface levels of the expressed receptors let alone attempt to adjust the expression levels of the various mutants to comparable levels to exclude that the effects observed would be simply a consequence of differential degrees of overexpression. This type of adjustment is absolutely critical to allow conclusions and should be included for all receptors (uPAR, integrins and mutants thereof).

> The referee points out that: 1) it is not clear whether the mechanisms described occurs at physiological levels of receptor and; 2) that it is critical to our conclusions that all the analyzed receptors are expressed a comparable levels.

1) As explained in detail in the response to Referee #1, the use of an over expression system has been critical for us to isolate and functionally characterize ligand-independent integrin signaling. We however fully realize the importance of documenting that similar signaling mechanisms may also be triggered in cells expressing physiological receptor levels. As elaborated also in the response to Referee #1, we have now included new data demonstrating ligand-independent integrin signaling downstream of uPAR in MDA-MB-231 cells that express (patho)physiological levels of the receptor. These cells do not adhere to VN^{RAD} and in order to do the experiments, we have had to use a stronger uPAR-substrate (an antibody). Despite this shortcoming, the experiment nevertheless clearly shows that uPAR-specific cell spreading also in this cell line is mediated by b_1 (new sup. Fig 2b).

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2) We have in this study systematically used the Flp-In T-REx 293 cell system (Invitrogen) for the expression of the different receptors and we believe that this is a significant strength of our study as it ensures consistent and highly comparable expression levels when comparing similar receptor variants. In this cell system, a single copy of the transfected expression cassette is recombined into the exact same genomic locus in the vast majority of the transfected cells thus resulting in the generation of a pool of isogenic clones. All the different receptor variants will therefore be expressed with identical transcriptional pressure and since we are working with a pool of isogenic clones we can also exclude clonal artifacts. We have used this expression system extensively in the past and have carefully validated that comparable expression levels are indeed achieved. In fact, we have measured the cell surface expression of more than 250 different uPAR variants by FACS analysis (Madsen et al, 2007) and found that all of these were expressed at very similar levels. Specifically, the cell lines expressing the $uPAR^{T54A}$ and $uPAR^{W32A}$ mutants employed in this study express 118% +/-13% and 97% +/-9% when compared to $uPAR^{WT}$ (mean +/-SD), respectively. We therefore believe that differences in uPAR-variant expression levels do not contribute to the observations and conclusions of this study.

We have employed the same cell system for the structure-function analysis of b_1, but in this case we can not accurately quantify the cell surface expression of the transgenic b_1 as we have no antibody that selectively identifies these b_1-chain. Well aware of this potential issue, we have taken particular care in documenting the functional activities of the b_1 variants also on a canonical substrate (FN) for which the effects of the different mutations are well described in the literature. Our findings on FN are indeed fully consistent with the accepted dogma for integrin-mediated cell adhesion and spreading where mutations impairing ligand binding or the talin/kindlin interaction completely impair receptor activity. For uPAR-mediated cell adhesion on VN^{RAD}, a very similar picture is observed with the important exception that the ligand-binding deficient (b_1^{D130A}) variant is active in promoting cell spreading. The fact that the same mutant is completely dead when the cells are seeded on FN strongly suggests that the activity we have assigned to this receptor is not caused by an aberrant (over)expression of this variant. To more directly assay if the different b_1-variants are expressed at comparable levels, we have now exploited the fact that the transgenic b_1-chains competes with endogenous b_1 for the generation of functional heterodimers. As the endogenous b_1, but not the transgenic chains, is recognized by the 4B4 antibody, a reduction in 4B4 surface staining therefore represents a proxy for effective competition with endogenous receptor. In this analysis, presented in new Sup. Fig. 3b, the expression of representative b_1-variants resulted in a comparable reduction in 4B4 staining indicating that they are expressed similarly and compete with endogenous receptor. Considering these evidences, we believe that it is very unlikely that differential expression of the different b_1 variants is a relevant confounding factor in this work.

For the different b_3-chains we have now conducted flow cytometry to document their cell surface expression levels and the data are presented in Sup. Fig. 4a. The expression level of b_3^{WT} and b_3^{119Y} are almost identical while the expression of the b_3^{Y2A} chain is mildly lower. Despite its reduced expression level, this receptor is still functionally active supporting cell adhesion on LM609 (Sup. Fig. 4e) and transducing ligand-independent b_1-dependent signaling (Fig. 6c). Also for b_3 integrin we therefore believe that differences in expression levels are unlikely to condition the validity of our conclusions.

The second major concern deals with the assessment of membrane tension. The AFM measurements that are used to demonstrate higher membrane tension in uPAR expressing cells are inconclusive. The authors perform indentation experiments on the cell body and show that there is no difference in the membrane tension within the control and uPAR-expressing cell. They then proceed to measure the lamella of the uPAR-expressing cell and find it two have a higher membrane tension than the cell body. From this they conclude that uPAR expressing cells have higher membrane tension. This conclusion is unfounded as the control cells apparently do not have a proper lamella and this is not measured. It has been show that the density of actin highly contributes to the elastic modulus of a cell. As the lamella is a thin membrane structure with extremely high actin content, it is very likely to be stiffer than the cell body. Therefore comparing lamella of uPAR expressing cells to cell body of control cells to conclude that membrane tension is increased is not correct. The AFM measurements should be performed from lamella of both cell types. If the control cells really do not for any lamella, these types of measurements cannot be carried out.

> We fully agree with this concern and have modified the manuscript accordingly. Clearly, the stiffness of uPAR-induced lamellipodia should be compared to the stiffness of a control lamellipodia and not to the stiffness of the cell body. Unfortunately, however, the control cells do not form

lamellipodia and we have therefore not been able to do the direct, and correct, comparison of stiffness. We included the AFM data in the manuscript primarily to document that lamellipodia induced by the uPAR/VN-interaction are associated with an increased stiffness similarly to what has previously been described for lamellipodia induced by canonical integrin signaling (Houk et al 2012). We still think this is a valid piece of information given the novelty of ligand-independent integrin-mediated signaling.

We have now moved the data to a supplementary figure (Sup. Fig. 5) and modified our description of the results in the text to underscore that the data do not demonstrate that uPAR induces membrane tension, nor that uPAR-induced lamellipodia are associated with a particularly high stiffness, but simply that lamellipodia triggered by uPAR are associated with an increased stiffness compared to the cell body as previously published for canonical integrin induced lamellipodia.

Importantly the AFM data and their interpretation are not a critical observation for our conclusion on the importance of membrane tension in ligand-independent integrin signaling, which is documented in different experiments.

Other points:

1) Does uPAR overexpression affect the cell surface levels of integrins? This should be addressed by FACS analysis.

> The overexpression of uPAR profoundly enhances cell spreading, migration and proliferation of 293 cells when these are cultured in serum-containing culture medium (i.e. in the presence of VN) and as shown for cell spreading in this manuscript (Fig. 1b) and in previously published works (Madsen et al, 2007; Pirazzoli et al, 2013), this biological activity of uPAR is strictly dependent on its direct interaction with VN. As suggested by the referee, it is indeed possible that the increased cell adhesion, caused by the interaction between uPAR and VN, affects the cell surface expression levels of integrins and that such altered levels might contribute to the biological effects of uPAR overexpression. However, using the same cells employed in the current study, we have previously shown that the cell surface levels of b_1, a_vb_5 and b_3 are not affected by overexpression of uPAR (Madsen et al, 2007). We have in the current study predominantly employed the T54A substitution variant of uPAR as this provides conditional VN-binding and we have now repeated the analysis of integrin cell surface expression levels also for these cells with and without the uPA treatment used to induce VN-binding (Sup. Fig. 1e). In this analysis, we find no evidence to suggest that induction of VN-binding to $uPAR^{T54A}$ affects the expression of relevant integrins and we therefore conclude that altered expression of integrins is very unlikely to contribute significantly to the findings presented in this study.

2) Adhesion experiment are shown as percentage of cells adhering on a single time point. The question is whether these cells that do not adhere are incapable of adhesion all together (for example because of not being viable) or whether adhesion is simply delayed. Adhesion time course experiments should be carried out, at least for the key experiments

> We routinely check viability of the cells we employ in the different assays and we have not noted particular problems in any of the cell lines employed in this study. This is substantiated by the fact that we observe very similar (basically 100%) cell adhesion to VN^{RAD} in the presence uPA (see for example Fig. 4e and 5a for the different integrin mutants). Also none of the cell lines display remarkable differences in cell adhesion to poly-D-lysine.

As pointed out by the referee another confounding factor could be differences in the kinetics of adhesion among the different cell lines that we might have overlooked in our end-point analysis of cell adhesion. To address this possibility, we have now conduced real-time analysis of cell adhesion for the different uPAR-variants seeded on VN (Sup. Fig. 1a) as well as for representative b_1-integrin variants (data presented in seeded on VN^{RAD}, FN and poly-D-lysine (Sup. Fig. 3c). The results of these analyses are very clear and show that the kinetics of cell adhesion is comparable for the different cell lines justifying the use of a single time-point.

3) In Western Blots for phospho-p130 Cas, total Cas should be also probed to exclude that overexpression or other manipulations affect total levels of p180Cas protein. The densitometric quantifications of phospho- Cas -levels should naturally

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> To exclude the possibility that our experimental conditions might affect the levels of total p130Cas, rather than its phosphorylation, we have now repeated almost all of the signaling experiments blotting for phosphorylated p130Cas, total p130Cas and vinculin (see Fig. 1d, 2d, 2c, 5b, 6a, 7a and S1b). In this analysis, we find no evidence to suggest that any of the employed experimental conditions affects the levels of total p130Cas. Our extensive use of vinculin as loading control is therefore justified and very unlikely to have compromise our quantifications. We have included the data from the new experiments in the quantitative analysis of independent experiments and the result remains the same even if the numbers are therefore now slightly different.

REFERENCES

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2nd Editorial Decision 23 May 2014

Thank you for your patience while your manuscript has been re-reviewed.

First of all, I would like to apologize for a mistake I made that prevented you from receiving the comments from referee #3. As you probably remember, in the interest of time I sent you a preliminary decision letter with the comments of referees #1 and #2. A technical issue in our system that we are working to correct, caused that I only became aware of the existence of this third report at the time you submitted your revised manuscript, obviously too late. In any case, I am appending below both the original report and the report on the revised manuscript from referee #3. As you will see, s/he is also positive towards your study and essentially, given that his/her original concerns were reasonably similar to those of the other referees, s/he is convinced that your manuscript is almost ready for publication provided that further discussion is added on the possibility of uPA interacting with integrins. Again, I sincerely apologize for this unusual mistake.

Along similar lines, referees #1 and #2 are not yet convinced that a role for integrins in force generation and substrate attachment could be conclusively ruled out by the experimental evidence provided, and suggest a few more experiments to further address this issue. Although it is our policy to allow a single round of revision, in this case, given the very positive consideration of your manuscript by the referees in terms of novelty and general interest, and taking into consideration that the experiments suggested are reasonable and follow the lines of the concerns raised in the first round of review, I would like to give you the opportunity to deal with these remaining issues.

Do not hesitate to contact me in case you have any further questions.

Thank you again for your patience and the opportunity to consider your work for publication. I look forward to the final version of your manuscript.

REFEREE REPORTS

Referee #1:

The manuscript has been well revised. I have only two very minor points, which, when addressed properly, would improve the paper.

1) Figure 2C, VN-RAD samples: The blot indicates absence of pCas in untreated cells but high levels of pCas after uPA stimulation, while on wildtype VN you have some pCas even in the absence of uPA. However, the quantification displays a similar fold change in Cas phosphorylation between VN-wt and VN-RAD. How does this discrepancy arise?

2) Stainings in Figure 3: The authors show β1 and α5 localization at the leading edge of protruding lamellipodia during cell spreading. However the integrins are not clustered in bigger adhesion structures. I would be interesting to show if the uPAR-mediated adhesion and integrin-medidated adhesion (e.g. on fibronectin) differ with respect to the formation of bigger adhesion structures. This figure could be improved in two ways: a) The authors could show β1 staining of 293 uPART54A seeded on fibronectin. b) An interesting aspect would be the distribution/localization of active integrins during uPAR-mediated adhesion and spreading. As Ferraris et al speculate that α5β1 is one of the major integrin implicated in this process this could be achieved by immunostaining with the 9EG7 antibody that recognized integrins in their active conformation.

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The authors have sufficiently addressed most criticisms and the manuscript has improved. One is still, however, left wondering how the cells produce sufficient amounts of force to rearrange the actin cytoskeleton in order to spread if the integrins on the plasma membrane are indeed not ligated. The fact that the cells spread only on stiff substrates (Fig. 7b), clearly indicates that the cells exert pulling force on the ECM in order to spread. GPI-anchored receptors obviously can mediate adhesion, but without a connection to the cytoskeleton they will not be able to generate sufficient force to remodel to actin cytoskeleton and spread, a process that is clearly happening in these cells (Fig. 3). It is unlikely that membrane tension would be sufficient to compensate for the lack of an ECM-actin connection. This is well demonstrated by genetic deletions of various adaptors that are essential for linking integrins to the actin cytoskeleton but not for integrin activation or ligand binding (for example vinculin, ILK, alpha-actinin). The deletion mutants of these proteins all fail to spread. The Supplementary movie 1 of a uPAR-dependent spreading cell bears striking resemblance to a fibroblast spreading on in an integrin-dependent manner. The authors state that the integrin clusters shown in Fig. 3a closely resemble clusters shown previously to contain unligated integrins. These clusters are, however, very difficult to distinguish from matrix-bound nascent adhesions (see for example Choi et al., Nat Cell Biol 2008; PMID:19160484).
In summary it is hard to believe that this type of spreading behavior would be not be generated by an ECM-actin linkage but purely through membrane tension as the authors speculate in the discussion section. The claim that the integrins indeed are not ligated is the key novel finding of the paper and therefore it should be convincingly shown. An alternative explanation for the finding is that the GPI-anchored receptor is required for the initial adhesion on VN, but once the cells have made contact they immediately secrete other ECM proteins such as Fibronectin, allowing integrins that are incapable of binding VN to eventually be ligated, to cluster and to establish a connection to the cytoskeleton.
In this regard it is unfortunate that the authors have not attempted to address the questions of Rev #1 regarding the lateral interactions of integrins and associated proteins with the GPI anchored receptors. This interaction would not have to be direct and could occur through adaptors. This important issue should be addressed by answering the following relative straight forward questions experimentally:

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I've gone through the revised version of that manuscript and am in principle satisfied with the point-to-point answers to the reviewers' questions.

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ORIGINAL REPORT

In this paper, Ferraris provide novel evidence on the functional interaction of the uPAR receptor and integrins in matrix-dependent adhesion, migration and signal transduction. The general take on this study is that integrin signal transduction may be activated via alternative, e.g. membrane tension-dependent pathways, in the absence of direct integrin/matrix interactions. This is demonstrated in vitro by the use of the uPAR system as an alternative matrix-coupling module, which apparently utilizes the integrin-dependent signal transduction machinery, even if integrin-matrix interactions are strongly abrogated. The study employs an impressive array of integrin mutants in conjunction with transfection-based assay systems to prove that point.

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2nd Revision - authors' response 18 July 2014

Referee #1:

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1) Figure 2C, VN-RAD samples: The blot indicates absence of pCas in untreated cells but high levels of pCas after uPA stimulation, while on wildtype VN you have some pCas even in the absence of uPA. However, the quantification displays a similar fold change in Cas phosphorylation between VN-wt and VN-RAD. How does this discrepancy arise?

> The referee correctly points out that the blot show in Figure 2C clearly indicates lower levels of pCas on VN^{rad} vs. on VN^{wt}, while the quantified data derived from multiple experiments (shown above the blots) reports similar levels of pCas on the two substrates.
We have now carefully investigated the matter to understand how this discrepancy arises. There are two reasons:
1) The VN^{RGD} and VN^{RAD} are reference conditions in several different experiments (i.e. not only in this "substrate" experiment) and the new data obtained during revision to validate total Cas vs. vinculin normalization were by mistake entered into the wrong table and the values in the graph therefore not updated accordingly (i.e. only the immunoblots were changed).
2) The "representative" western blot shown in the figure was taken from the experiments done for the revision where total Cas was also analysed. In that particular experiment the pCas phosphorylation on VN^{RAD} in the absence of uPA is particularly low as compared to the "average" experiment represented by the quantified data. This is really just experimental variability and underscores the importance of quantitative analysis of independent experimental replicates that we believe to have employed quite extensively in this study. We hope that the referee will understand that selecting a truly "representative" experiment is difficult when far most of the experiments were done prior to the requested total pCas normalization.

The question however remains: is Cas phosphorylation lower or higher on VN^{RAD} as compared to VN^{WT}? To address this issue we have now pooled all the data we have on these experimental conditions and Figure 2C have been updated accordingly. The graph now summarizes 16, 6, 8 and 7 independent experiments for VN^{WT}, VN^{RAD}, poly-lysine and FN, respectively.
In average pCas phosphorylation is a bit higher on VN^{WT} than on VN^{RGD}, but the difference is presumably not significant, nor interesting.

2) Stainings in Figure 3: The authors show $\beta1$ and $\alpha5$ localization at the leading edge of protruding lamellipodia during cell spreading. However the integrins are not clustered in bigger adhesion structures. I would be interesting to show if the uPAR-mediated adhesion and integrin-medidated adhesion (e.g. on fibronectin) differ with respect to the formation of bigger adhesion structures. This figure could be improved in two ways: a) The authors could show $\beta1$ staining of 293 uPART54A seeded on fibronectin. b) An interesting aspect would be the distribution/localization of active integrins during uPAR-mediated adhesion and spreading. As Ferraris et al speculate that $\alpha5\beta1$ is one of the major integrin implicated in this process this could be achieved by immunostaining with the 9EG7 antibody that recognized integrins in their active conformation.

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> We have now done the experiments suggested by the referee.
In the new Figure 3, Panel A, the distribution of total (mAb K20) and active b1 (mAb 9EG10) integrin is presented for uPAR-expressing cells seeded on FN and VN^{RAD}. As expected, a strong coincident staining is observed for total and active b1 in focal contacts when cells are seeded on FN. On VN^{RAD}, focal contact structures are absent and b1 predominantly localizes close to the leading edge of lamellipodia. The staining for active b1 on this substrate is less clear and there is no apparent co-localization with total b1 close to the leading edge. It has been established that 9EG10 preferentially recognizes ligand-occupied active b1 (Bazzoni et al. JBC 1995: *270* 25570-7, Askari et al. JCB 2010: *188* 891-903) and this result therefore does not demonstrate that the b1 observed close to the leading edge is inactive.

To address the importance of the a5, previously only deduced only from its localization, we have now knocked down the a5 subunit and assayed the consequence for cell spreading on VN^{RAD} (New Sup. Fig 4c). The data experimentally confirms the importance of the a5b1 heterodimer in the process.

Referee #2:

The authors have sufficiently addressed most criticisms and the manuscript has improved. One is still, however, left wondering how the cells produce sufficient amounts of force to rearrange the actin cytoskeleton in order to spread if the integrins on the plasma membrane are indeed not ligated. The fact that the cells spread only on stiff substrates (Fig. 7b), clearly indicates that the cells exert pulling force on the ECM in order to spread. GPI-anchored receptors obviously can mediate adhesion, but without a connection to the cytoskeleton they will not be able to generate sufficient force to remodel to actin cytoskeleton and spread, a process that is clearly happening in these cells (Fig. 3). It is unlikely that membrane tension would be sufficient to compensate for the lack of an ECM-actin connection. This is well demonstrated by genetic deletions of various adaptors that are essential for linking integrins to the actin cytoskeleton but not for integrin activation or ligand binding (for example vinculin, ILK, alpha-actinin). The deletion mutants of these proteins all fail to spread.
> The referee argue that membrane tension is insufficient to compensate for the lack of an ECM-actin connection as deletion of different adaptor proteins all lead to impaired cell spreading, but not impaired integrin activation and ligand binding. Our data are fully consistent with these previous findings as disruption of the molecular clutch on the cytoplasmic side of the plasma membrane (in our study through mutation of the NPxY-motifs or knock-down of talin) invariably prevents cell spreading. The connection of actin to the plasma membrane (via adaptors and the cytoplasmic tails of integrins) therefore seems to be ubiquitously critical for cell spreading. This makes sense, as it is difficult to imagine how force transmission may occur without this connection. Importantly, however, we are in this work disrupting the ECM-actin connection *outside* the cell membrane by preventing the interaction between the integrin and the ECM. This has been done in numerous times previously and invariably prevents cell spreading on defined integrin ECM-substrates. Again, this makes sense, as it is difficult to imagine how a cell can spread on a substrate if it does not bind to it. However, differently from previous studies we have here restored cell adhesion *without* restoring integrin binding to the ECM. This decoupling has allowed us to show that the integrins remain a critical component of the molecular clutch and that membrane tension in this situation becomes an important player.

We here present a couple of different cases where membrane tension strongly modulates cell spreading induced by non-integrin adhesion receptors. We don't think, postulate nor speculate that membrane tension *alone* is able to do any of this. We fully agree with the referee that it is very unlikely that membrane tension can directly couple the ECM to the cytoskeleton. However, we can't see why membrane tension should not be able to affect the mechanical coupling between close-by membrane receptors (i.e. uPAR and integrins).

The Supplementary movie 1 of a uPAR-dependent spreading cell bears striking resemblance to a fibroblast spreading on in an integrin-dependent manner. The authors state that the integrin clusters shown in Fig. 3a closely resemble clusters shown previously to contain unligated integrins. These clusters are, however, very difficult to distinguish from matrix-bound nascent adhesions (see for example Choi et al., Nat Cell Biol 2008; PMID:19160484).

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In summary it is hard to believe that this type of spreading behavior would be not be generated by an ECM-actin linkage but purely through membrane tension as the authors speculate in the discussion section. The claim that the integrins indeed are not ligated is the key novel finding of the paper and therefore it should be convincingly shown. An alternative explanation for the finding is that the GPI-anchored receptor is required for the initial adhesion on VN, but once the cells have made contact they immediately secrete other ECM proteins such as Fibronectin, allowing integrins that are incapable of binding VN to eventually be ligated, to cluster and to establish a connection to the cytoskeleton.
In this regard it is unfortunate that the authors have not attempted to address the questions of Rev #1 regarding the lateral interactions of integrins and associated proteins with the GPI anchored receptors. This interaction would not have to be direct and could occur through adaptors. This important issue should be addressed by answering the following relative straight forward questions experimentally:

-Does uPAR colocalize with the integrin clusters in spreading cells?
> The 293/uPAR cells express to so high levels of uPAR that fluorescence co-localization experiments do not make much sense (uPAR is everywhere on the cell surface). We have therefore conducted the suggested analysis in MDA-MB-231 cells seeded on FN and VN and the data are shown in new Sup Fig. 3B. On both substrates uPAR and b1 are localized in the same sub-cellular location (i.e. concentrated in protrusions). There is some co-localization, but it is not striking and in our opinion not enough to suggest/support a direct interaction between the two receptors.

It is not true that we have not addressed the question of Rev#1 regarding lateral interactions between uPAR and b1. We did include additional experiments where we found that mutating the presumed uPAR binding site in b1 (the S227A substitution, see Fig 4c) was without any relevant effect on the measured process. We believe that the methods we have employed here (structure-function analysis of the integrin and complementation analysis of the adhesion receptor) are very potent and reliable tools as compared to classical vicinity assays for membrane proteins (co-immunoprecipitation and co-localization).

-Do these spreading cells display focal contacts as visualized by for example talin or paxillin?
> Data addressing this point are now presented in the new Figure 3. As expected from the absence of integrin binding sites in VN^{RAD} we do not observe structures consistent with focal contacts on this substrate.

-Is the spreading dependent on myosin activity?
> New data presented in Figure Sup. 2b shows that cell spreading on VN^{RAD} is not inhibited by Blebbistatin, indicating that the process is largely powered by actin polymerization.

-Does the beta1 K218/D130A bind VNRAD in the absence of uPAR?
> We have not done this experiment directly (i.e. with cells expressing no uPAR) as we find it unlikely that the D130 substitution could act as a gain-of-function mutation allowing $b1^{D130}$ to bind VN^{RAD} when $b1^{wt}$ does not even bind VN^{wt}. The question is however indirectly addressed in Sup. Fig. 5c where no measurable adhesion of the $293/uPAR^{T54A}/b1^{D130}$ cells is observed to VN^{RAD} in the absence of uPA.

-Can the authors exclude that the cells deposit minor amounts of Fibronectin or Collagen during the 30 min of assay time that would allow VN-independent integrin ligation and signaling?
> No - we can't exclude this possibility and one of our initial hypotheses was indeed that cell adhesion triggered by the $uPAR/VN^{RAD}$-interaction would permit the cells to deposit some type of provisional ECM and subsequently spread by the canonical ligand-dependent integrin mechanism. This would indeed conveniently explain the peculiar b1-dependence of cell spreading on VN^{RAD}. To address this possibility, we did look for fibronectin and laminin deposits by immunofluorescence, but found none consistent with the fairly rapid kinetics of cell spreading observed in this model system (cell spreading may be appreciated just seconds subsequent to the induction of the uPAR/VN-interaction by treatment with uPA – see supplementary Movie 1).

We later abandoned the hypothesis altogether because of the observation that the ligand binding dead $b1^{D130}$ is active in supporting cell spreading. Even if the cells should deposit ECM rapidly during the uPAR-mediated adhesion, this integrin variant would not be able to bind it. The same holds true for $b3^{D119Y}$.

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We therefore conclude that the possible deposition of ECM occurring in the early phases after cell adhesion is not a significant player in the phenomena characterized here.

Referee #3:

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> With reference to published work demonstrating that uPA may interact directly with integrins through the kringle domain (Tarui 2006) the referee correctly points out that a physical bridging of uPAR and integrins may occur via this domain and that this would represent a “bypass” mechanism resulting in a different model.

We believe that several findings already presented in this study indicate that this bypass mechanism is very unlikely to play any significant role. Rather than discussing these extensively, we have now addressed the issue directly (see result section and Sup. Fig 2a-b). We here show that the uPAR binding GFD-domain of uPA is equally efficient, or maybe even better, in inducing pCas phosphorylation and cell spreading on VN^{RAD}. The GFD domain of uPA does not contain the kringle domain and therefore cannot form any bridge between uPAR and integrins according to the data published by Tarui et al.

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In this paper, Ferraris provide novel evidence on the functional interaction of the uPAR receptor and integrins in matrix-dependent adhesion, migration and signal transduction. The general take on this study is that integrin signal transduction may be activated via alternative, e.g. membrane tension-dependent pathways, in the absence of direct integrin/matrix interactions. This is demonstrated in vitro by the use of the uPAR system as an alternative matrix-coupling module, which apparently utilizes the integrin-dependent signal transduction machinery, even if integrin-matrix interactions are strongly abrogated. The study employs an impressive array of integrin mutants in conjunction with transfection-based assay systems to prove that point.

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