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Actomyosin bundles serve as a tension sensor and a platform for ERK activation

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Barbara Pauly

1st Editorial Decision   17 July 2014

Thank you for the submission of your research manuscript to our editorial offices. We have now received the three enclosed reports on it. As you will see, the referees agree on the potential interest of the manuscript, but they have also all raised a number of substantial concerns about the study that would need to be addressed prior to publication.

Most importantly, all three reviewers agree that additional mechanistic insights are needed to substantiate the claim that tension alone is sufficient to activate ERK signaling (as most clearly highlighted in the report of referee 2). In addition, reviewer 2 is not convinced that the current dataset conclusively demonstrates that the cells have indeed experienced force and additional controls would be needed to show this. Reviewer 3 points out that it would be interesting to analyze the dynamics of ERK activation by laser cutting in a live cell set-up. Both referees 1 and 3 also feel that additional insights into the functional consequences of the tension-mediated ERK activation are needed and referee 1 feels that stronger proof for the activation of ERK would be required, in addition to the immunostaining approach.

From the analysis of these comments it is clear that publication of your manuscript in our journal can only be considered after significant revision. But given the potential interest of your study and the reviewers' constructive suggestions on how to improve it, we would like to give you the opportunity to address the referee concerns and would be willing to consider a revised manuscript with the understanding that the main issues raised by our reviewers must be fully addressed.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

This manuscript proposes the interesting idea that stress fibers may contribute to tension-sensitive MAPK/ERK signaling by providing the platform for ERK phosphorylation (and hence activation). The authors show evidence that active pERK (but less so the total pool of ERK) localizes to stress fibers in a tension-sensitive fashion. They combine manipulation of endogenous tension (inhibiting myosin) and exogenous stretch of the cells as manoeuvres to alter tension. Overall, this is a somewhat phenomenological paper (e.g. it does not identify a mechanism for pERK to localize to stress fibers nor is the functional significance clear), but one that is potentially very interesting.

Major comments.

1. The notion that specifically pERK might localize to stress fibers is interesting, but surprising. The conclusion is founded on immunolocalization for pERK (the biochemical assays shown in Fig 1 simply tell us that ERK activation is myosin-sensitive, they don't tell us where the active ERK may localize).

I think that it is therefore important to validate the immunostaining, to exclude bleedthrough artifacts or cross-reactivity of the Ab with another protein or epitope found on SF. As the whole paper is based on this IF assay, I think it is essential to perform these controls:
a. Label with phalloidin, but not pERK, to test the bleedthrough.
b. Block ERK activation pharmacologically and test staining.
c. Deplete p42/44 ERK by RNAi and test staining.
In addition, which pERK antibody is used for the data in the main figures? (Presumably the mouse, as its staining seems better?)

2. I am not sure that I fully understand what the authors mean when they refer to activation of ERK on SF? Are they proposing that ERK phosphorylation occurs on the SF? (This is my reading of their discussion, which would fit with their notion that SF may be tension sensors for ERK activation). This would be surprising, as the pathway from mitogens is often thought to be at the plasma membrane. Or do they mean that, once phosphorylated, ERK localizes to SF. The latter would also fit their data. I think that they need to clarify this.

Referee #2:

This study reports on localization of p-ERK to stress fibers and the requirement for force in this activation process. The authors conclude that actomyosin bundles themselves can act as a signaling platform for tension-sensitive signaling events. The role of force in controlling local signaling events is now very well recognized and plays a key role in a wide range of biological processes. Active ERK has previously been shown to localise to adhesions/stress fibers, be dependent upon active Rho signaling pathways and play nuclear and non-nuclear signaling roles, so the current study in not novel conceptually. The mechanics ideas being tested here are potentially of interest, but the current study fails to convincingly provide evidence that stress fibres can act as a template in the absence of any other signaling or adaptor proteins or attempt to define any of the upstream factors are required to regulate local active Erk. Thus the current manuscript falls short of delivering any novel information to this field. The specific issues are detailed below:
- Fig 1: the localisation of Erk to stress fibres, activation of Erk by mechanical stretch and requirement for Rho signaling have all been previously shown, so this figure offers nothing novel. Moreover, the quantification of signal intensity in B and D is now described in the methods, but if

any signal is 'normalised' to the DMSO control, why then do these bars have errors associated if they are always set to 1? There is a clear difference in intensity between DMSO and +Blebb Erk staining, as well as p-Erk from the images shown that is not evident from the graph. Hence there is little confidence, or indeed benefit, in the way the authors have quantified this data. Why are there two bands for total Erk (presumably p44/42) and only one of p-Erk in 1E? Is this because only p42 is phosphorylated in these cells? The changes being proposed here in Erk activation with stretch and Blebbistatin and very small - and no stretch alone is included. Why did the authors not use serum starvation to reduce to basal conditions? Does inactive Erk also localize to stress fibres in the absence of any activation signal? All of the images provided in this manuscript are widefield, meaning no resolution on Z-axis is provided. This would also be essential to better define the subcellular distribution of p-Erk throughout the study.

- The total Erk signal appears partly in the nucleus in Fig 1C - this then never appears again, but clearly Erk is a very well known nuclear translocator upon activation. One of the key questions here is why these cells fail to show nuclear translocation of p-Erk under any of the conditions tested? The authors never address this or discuss at any point the factors that may control nuclear vs SF p-Erk association. These sorts of mechanistic insights would be essential to give the present study a much-needed novel angle.

- Fig 2: why is there so much non-cell associated p-Erk staining in panels A? And why are there so many stress fibres remaining in cells in both panels given that they are supposed to have been treated with Blebb? These cells look like controls. There is also little confidence that the authors are able to conclude that the arrowed cell in the bottom panels of 2A has experienced force (not least as the cell above it has not re-oriented or altered SF at all). Such experiments must be done live to be able to conclude anything regarding a stretch-response. At present this figure merely demonstrates exactly what Figure 1 showed (and others have previously published) - ie: p-Erk is localized on SF - and that this is also the case if the SF happen to be oriented within the direction of force. No direct evidence of force response is provided, merely a correlation.

- Similar concerns relate to figure 3 - these measurements are based on analysis of fixed images of cells and assumptions made about local force production by individual SF. Moreover, the images in Fig3 of the p-Erk are very difficult to see as they are masked by the arrows, but it appears that in both control and Blebb treated cells, the level of p-Erk simply correlates with the intensity of F-actin labelling - ie: the larger/wide the SF, the higher the p-Erk signal. This again provides no evidence of a force-induced response rather that larger SF can recruit (directly or indirectly) more p-Erk, and therefore provides no additional insight into this process.

- The authors use an additional micro-pattern model in Figure 4 that again shows that p-Erk is recruited to SF but again provides no additional insight into the regulatory mechanisms that govern this process. Thus it is not clear how or what this additional datatset adds to the manuscript.

Referee #3:

In this short manuscript, the authors report that the MAP-kinase ERK is phosphorylated in actin stress fibers. By using blebbistatin for myosin II inhibition, cell stretching and pillar assays, they prove that this activation occurs in a tension-dependent manner.

In general, this is an interesting observation that deserves publication, although a more mechanistic understanding is missing. I have only a small number of minor comments.

First the classical stress fibers investigated here are usually striated and I wonder if the ERK-activation is variable along the stress fiber. If possible, the authors should stain for myosin II and alpha-actinin and compare with the ERK-signal, eg in a line plot. I also wonder if more can be said regarding ERK-activation in focal adhesions.

Second I wonder if it can be shown that the whole signaling cascade is active; showing phosphorylated ERK does not prove that the tension has a functional effect on the cells.

Third I find that a few references are missing that earlier have provided insight into the relation between tension and protein localization using laser cutting. Apart from Ref. 54 (Kumar et al. Biophysical Journal 2006), Colombelli et al. Journal of Cell Science 2009 and Tanner et al.

Biophysical Journal 2010 should be cited and discussed. In general, laser cutting would also be a great tool to address the dynamics of ERK-activation.

1st Revision - authors' response   03 November 2014

Response to the Reviewer 1

We thank the reviewer very much for the helpful comments. We have extensively modified our manuscript according to the comments (modifications are colored in red in the revised manuscript). Below are our point-by-point responses to the Reviewer's comments.

This manuscript proposes the interesting idea that stress fibers may contribute to tension-sensitive MAPK/ERK signaling by providing the platform for ERK phosphorylation (and hence activation). The authors show evidence that active pERK (but less so the total pool of ERK) localizes to stress fibers in a tension-sensitive fashion. They combine manipulation of endogenous tension (inhibiting myosin) and exogenous stretch of the cells as manoeuvres to alter tension. Overall, this is a somewhat phenomenological paper (e.g. it does not identify a mechanism for pERK to localize to stress fibers nor is the functional significance clear), but one that is potentially very interesting.

Point 1

The notion that specifically pERK might localize to stress fibers is interesting, but surprising. The conclusion is founded on immunolocalization for pERK (the biochemical assays shown in Fig 1 simply tell us that ERK activation is myosin-sensitive, they don't tell us where the active ERK may localize).
I think that it is therefore important to validate the immunostaining, to exclude bleedthrough artifacts or cross-reactivity of the Ab with another protein or epitope found on SF. As the whole paper is based on this IF assay, I think it is essential to perform these controls:
a. Label with phalloidin, but not pERK, to test the bleedthrough.
b. Block ERK activation pharmacologically and test staining.
c. Deplete p42/44 ERK by RNAi and test staining.
In addition, which pERK antibody is used for the data in the main figures? (Presumably the mouse, as its staining seems better?)

Response 1
Thank you for the critical comments. Although localization of ERK and pERK to stress fibers has been repeatedly reported (for example, Zuckerbraun et al., 2003, Circulation 108:876; Pritchard et al., 2004, Moll Cell Biol 24:5937; Appel et al., 2010, Mol Biol Cell 21:1409; Vetterkind et al., 2012, PLoS ONE 7:e30409), we performed the suggested control experiments and added their results as follows.
a. Label with phalloidin, but not pERK, to test the bleedthrough.
By staining cells for either pERK, ERK or F-actin, we confirmed that the bleedthrough effect between different fluorescence channels was negligible. This point was mentioned in Supplementary Information, Page 3, Line 6-7, and the data was shown in Fig. S17.
b. Block ERK activation pharmacologically and test staining.
Since it has been reported that Src and FAK are involved in mechanical stimulus-induced ERK phosphorylation (Li et al., 2007, J Cell Biochem 100:129; Li et al., 2009, PLoS ONE 4:e7489; Hong et al., 2010, Mol Cell Biochem 335:263; Shih et al., 2011, J Bone Miner Res 26:730), we inhibited them pharmacologically and tested phosphorylated ERK staining. Treatment with the Src or FAK inhibitor largely reduced ERK phosphorylation in immunoblotting as reported previously, and this was associated with a decrease in immunofluorescence intensity of phosphorylated ERK on stress fibers. This point was mentioned in the main text, Page 8, Line 12-14, and the results were shown in Fig. S15.
c. Deplete p42/44 ERK by RNAi and test staining.
Expression of ERK1 or ERK2 was depleted in HFF cells using shRNA. ERK2 was preferentially phosphorylated in HFF cells compared with ERK1, and depletion of ERK2 expression, but not

ERK1 expression, significantly declined immunofluorescence intensity of pERK on stress fibers. These points were mentioned in the main text, Page 4, Line 21-23. The results were shown in Fig. S5, and the method for the shRNA-mediated depletion of ERK1 or ERK2 expression was described in Supplementary Information, Page 3, Line 21-30.

We used the mouse monoclonal anti-pERK antibody for all immunostaining experiments for pERK except for those shown in Fig. S3. This point was clarified in Supplementary Information, Page 2, Line 11-13.

Point 2

I am not sure that I fully understand what the authors mean when they refer to activation of ERK on SF? Are they proposing that ERK phosphorylation occurs on the SF? (This is my reading of their discussion, which would fit with their notion that SF may be tension sensors for ERK activation). This would be surprising, as the pathway from mitogens is often thought to be at the plasma membrane. Or do they mean that, once phosphorylated, ERK localizes to SF. The latter would also fit their data. I think that they need to clarify this.

Response 2

As the reviewer pointed out, canonical ERK phosphorylation mediated by G-protein coupled receptor signaling occurs at the plasma membrane. On the other hand, phosphorylation of actin-associated ERK has also been reported (Vetterkind et al., 2013, Cell Commun Signal 11:65). If recruitment of phosphorylated ERK to stress fibers was increased, the amount of total ERK on the fibers would also be increased. However, we observed that myosin II activity increased the amount of phosphorylated ERK on stress fibers without an increase in the amount of total ERK. It is therefore unlikely that the myosin II-dependent increase in phosphorylated ERK on stress fibers is mediated by increased recruitment of phosphorylated ERK to the fibers. Rather, it is more likely that ERK localizes on stress fibers independently of myosin II activity, and that it is phosphorylated in a myosin II-dependent manner. We discussed these points in the main text, Page 4, Line 23-31, and Page 8, Line 9-12.

Response to the Reviewer 2

We thank the reviewer very much for the helpful comments. We have extensively modified our manuscript according to the comments (modifications are colored in red in the revised manuscript). Below are our point-by-point responses to the Reviewer's comments.

General comments

This study reports on localization of p-ERK to stress fibers and the requirement for force in this activation process. The authors conclude that actomyosin bundles themselves can act as a signaling platform for tension-sensitive signaling events. The role of force in controlling local signaling events is now very well recognized and plays a key role in a wide range of biological processes. Active ERK has previously been shown to localise to adhesions/stress fibers, be dependent upon active Rho signaling pathways and play nuclear and non-nuclear signaling roles, so the current study in not novel conceptually. The mechanics ideas being tested here are potentially of interest, but the current study fails to convincingly provide evidence that stress fibres can act as a template in the absence of any other signaling or adaptor proteins or attempt to define any of the upstream factors are required to regulate local active Erk. Thus the current manuscript falls short of delivering any novel information to this field. The specific issues are detailed below:

Response to the general comments

As the reviewer mentioned, it has been revealed that force plays critical roles in controlling local signaling events. Focal adhesion (FA) is a well studied example of subcellular structures at which mechanical inputs are transduced into biochemical signals. Stress fibers have been recognized to be involved in regulating mechanotransduction at FAs by transmitting force to FAs. By contrast, the possibility that stress fibers per se work as a mechanotransduction platform has not been experimentally tested. Localization of phosphorylated ERK (pERK) to stress fibers has been reported, and RhoA signaling is reportedly involved in this. However, since the quantitative relationship between force and ERK phosphorylation on stress fibers has not been studied, the actual

role of force on stress fibers in ERK phosphorylation remains unclear. In this study, we altered force on stress fibers by myosin II inhibition and mechanical stretching of the extracellular substrate, and quantitatively showed the relationship between tensile force and the pERK amount on stress fibers by using mechanical modeling of stretched stress fibers and microforce sensor arrays. To make the results more convincing, we added a new set of data in the revised manuscript based on the reviewer's comments (please see below). Furthermore, we showed that serum-mediated signaling and/or Src-FAK signaling is required for force-induced ERK phosphorylation on stress fibers. Base on the results, we proposed a model that stress fibers serve as a platform for integrating chemical signal and mechanical force to achieve ERK phosphorylation, which, we strongly believe, is conceptually novel.

Point 1

Fig 1: the localisation of Erk to stress fibres, activation of Erk by mechanical stretch and requirement for Rho signaling have all been previously shown, so this figure offers nothing novel.

Response 1

As the reviewer pointed out, it has been revealed that mechanical stretch and RhoA activation, both of which potentially increase tension in the actin cytoskeleton-focal adhesion complex, cause ERK activation. However, information of where and how ERK is mechanically activated is elusive. Although localization of ERK to stress fibers has been repeatedly reported, it is totally unclear whether mechanical tension induces ERK activation on stress fibers on site. In Fig. 1 of this study, we showed that myosin II activity increased the relative amount of pERK, but not total ERK, against that of F-actin on stress fiber (please see also **Response 3**). This shows that ERK localizes on stress fibers independently of myosin II activity, and it is phosphorylated on the fibers in a myosin II-dependent manner. These results bring the novel insight that stress fibers work as a platform for myosin II-dependent ERK activation. Together with the result that the role of myosin II in increasing pERK on stress fibers could be substituted with tensile force induced by externally applied stretching (Fig. 2), we experimentally demonstrated, for the first time, that mechanical tension in stress fibers is the factor that increases pERK on SFs. These points were mentioned in the main text, Page 4, Lines 4-7 and 23-31, and Page 7, Line 7-11.

Point 2

Moreover, the quantification of signal intensity in B and D is now described in the methods, but if any signal is 'normalised' to the DMSO control, why then do these bars have errors associated if they are always set to 1?

Response 2

Each sample value (a_i) in the control (DMSO) population was normalized with respect to the mean value of the control population. The control population after the normalization $\{\dfrac{a_i}{\dfrac{1}{n}\sum\limits_{j}^{n} a_j}\}$ has a deviation, even though its mean value is of course one. To clarify this point, we made a modification in the legend of Fig. 1 (Page 15, Line 8-9).

Point 3

There is a clear difference in intensity between DMSO and +Blebb Erk staining, as well as p-Erk from the images shown that is not evident from the graph. Hence there is little confidence, or indeed benefit, in the way the authors have quantified this data.

Response 3

Although blebbistatin treatment decreased the number and fluorescence intensity of stress fibers (please see also Fig. S6), ERK was apparently localized on residual fibers in blebbistatin-treated cells. For clarification, we added arrows that denote localized ERK on residual fibers in Fig. 1C. By contrast, pERK intensity on stress fibers was decreased much more obviously compared with ERK intensity. To make the quantification analysis more convincing, the analysis method was changed. The difference in the amount of F-actin in each stress fiber was compensated by calculating the intensity ratio of phorphorylated ERK (pERK) or ERK against F-actin on individual stress fibers. The result was that the blebbistatin treatment decreased the pERK/F-actin ratio, but not the ERK/F-

actin ratio, on stress fibers. We modified the main text (Page 4, Line 23-28), Fig. 1B and C, and their figure legends (Page 15, Lines 5-6 and 6-8), accordingly. The new data of Fig. S6 was also added.

Point 4

Why are there two bands for total Erk (presumably p44/42) and only one of p-Erk in 1E? Is this because only p42 is phosphorylated in these cells?

Response 4

Yes, p42 MAPK (ERK2) was preferentially phosphorylated in these cells. This was conformed by depleting expression of either p44 (ERK1) or p42 (ERK2) MAPK (Fig. S5A). We mentioned this in the main text, Page 4, Line 21-23. For clarification, we added labels of ERK1 and ERK2 to bands in Fig. S7A (Fig. 1E in the original manuscript). The data of shRNA-mediated depletion of ERK1 or ERK2 expression (Fig. S5A) and its figure legend (Supplementary Information, Page 6, Line 19-24) were added. The method for the shRNA-mediated depletion of ERK1 or ERK2 expression was described in Supplementary Information, Page 3, Line 21-30.

Point 5

The changes being proposed here in Erk activation with stretch and Blebbistatin and very small - and no stretch alone is included.

Response 5

The densitometric analysis in Fig. S7A (Fig. 1E in the original manuscript) showed that the pERK/ERK ratio was decreased to $58\pm14\%$ of the control value upon the blebbistatin treatment. The pERK/ERK ratio was increased to the control level by mechanical stretching (Fig. S7A), suggesting that the blebbistatin-sensitive population of pERK is indeed regulated by mechanical force. Notably, the obtained percentage value in immunoblotting was in good agreement with the value obtained from the immunofluorescence experiment; the fluorescence intensity ratio of pERK/ERK for cells double-stained for pERK and ERK was decreased to $51\pm15\%$ of the control value by the blebbistatin treatment (Fig. 1D). Thus, while there is a blebbistatin-insensitive population of pERK, 40-50% of ERK phosphorylation is dependent on mechanical force. Since the force-induced ERK activation was associated with increased phosphorylation of RSK, a major effector of ERK (Fig. S12A), it is likely that this population of phosphorylated ERK is large enough for activating the downstream signal. We mentioned these points in the main text, from Page 4, Line 33 to Page 5, Lines 3, and Page 6, Line 18-19, and added the new data of Fig. S12A and its figure legend in Supplementary Information, Page 8, Line 13-19.

Based on the reviewer's suggestion, we examined the effect of stretching of cells without blebbistatin treatment. In cells not treated with blebbistatin, stretching did not increase ERK phosphorylation (Fig. S7B), implying that the endogenous level of myosin II-based tension is high enough for full phosphorylation of the force-responsive population of ERK. We mentioned these points in the main text, Page 6, Line 22-25, and added the new data of Fig. S7B and its figure legend in Supplementary Information, Page 7, Line 7-9.

Point 6

Why did the authors not use serum starvation to reduce to basal conditions? Does inactive Erk also localize to stress fibres in the absence of any activation signal?

Response 6

The authors appreciate these valuable comments, by which we could find an important factor for the mechano-dependent ERK phosphorylation on stress fibers. We examined the effect of serum starvation, and found that ERK was localized to the actin cytoskeleton in serum-starved cells, but substratum stretching did not cause an increase in ERK phosphorylation under serum starvation (Fig. S16). This suggests that while ERK localization to the actin cytoskeleton does not require serum stimulation, serum-mediated signaling and cytoskeletal tension are both involved in ERK phosphorylation. Thus, stress fibers are likely to serve as a platform for integrating these chemical and mechanical signals to induce ERK phosphorylation. These points were mentioned in the main text, Page 8, Line 17-22, and the new data of Fig. S16 and its figure legend (Supplementary Information, Page 9, Line 11-21) were added.

Point 7

All of the images provided in this manuscript are widefield, meaning no resolution on Z-axis is provided. This would also be essential to better define the subcellular distribution of p-Erk throughout the study.

Response 7
We examined subcellular distributions of pERK as well as ERK using a confocal microscope, and observed clear localization of pERK and ERK on stress fibers (Fig. S1A-C). Importantly, confocal and wide-field observations gave a similar result of quantification analyses of pERK and ERK intensities on stress fibers (Fig. 1B and Fig. S1D). Thus, lower Z-axis resolution with a wide-field microscope does not affect results and conclusions in this study. We, therefore, keep using the original data obtained by wide-field observations. These points were mentioned in the main text, Page 4, Line 28-29, and the new data of Fig. S1 and its figure legend (Supplementary Information, Page 5, Line 17-28) were added. Information of the confocal microscope was described in Supplementary Information (Page 3, Line 7-9).

Point 8
The total Erk signal appears partly in the nucleus in Fig 1C - this then never appears again, but clearly Erk is a very well known nuclear translocator upon activation. One of the key questions here is why these cells fail to show nuclear translocation of p-Erk under any of the conditions tested? The authors never address this or discuss at any point the factors that may control nuclear vs SF p-Erk association. These sorts of mechanistic insights would be essential to give the present study a much-needed novel angle.

Response 8
We examined the nuclear distribution of pERK. While pERK was distributed throughout cytoplasm and nuclei in control cells, pERK was excluded from nuclei upon the blebbistatin treatment (Fig. S8). This result is consistent with the notions that myosin II activity increases phosphorylation of ERK, and that nuclear translocation of ERK is facilitated by its phosphorylation. The relationship between stress fiber-mediated ERK phosphorylation and its nuclear translocation is unclear at present. However, this point is beyond the scope of this study, and needs to be revealed in future studies. We mentioned these points in the main text, Page 5, Line 4-6, and added the new data of Fig. S8 and its figure legend in Supplementary Information, Page 7, Line 11-14.

Point 9
Fig 2: why is there so much non-cell associated p-Erk staining in panels A?

Response 9
When immunostaining is carried out on fibronectin-coated, PDMS-based stretch chambers, fluorescent dots adhering to the chamber surface outside cells sometimes appear, even though we do not know the actual reason causing this. However, these dots did not interfere with analyses of fluorescence intensities in cell regions. Rather, they provided internal controls for focus and exposure. To avoid misleading, we substituted original images with the ones with fewer dots in Fig. 2A.

Point 10
And why are there so many stress fibres remaining in cells in both panels given that they are supposed to have been treated with Blebb? These cells look like controls.

Response 10
Consistently with the results reported previously (Hirata et al., 2008, J Cell Sci 121:2795; Hirata et al., 2014, Am J Physiol Cell Physiol 306:C607), human foreskin fibroblasts had residual stress fibers even after the treatment with 100 μM blebbistatin for 30 min. However, the number of stress fibers and F-actin intensity on each fiber were largely decreased upon the blebbistatin treatment (Fig. S6). These points were mentioned in the main text, Page 4, Line 23-27, and the new data of Fig. S6 and its figure legend (Supplementary Information, Page 6, Line 26-31) were added.

Point 11
There is also little confidence that the authors are able to conclude that the arrowed cell in the bottom panels of 2A has experienced force (not least as the cell above it has not re-oriented or altered SF at all). Such experiments must be done live to be able to conclude anything regarding a

stretch-response. At present this figure merely demonstrates exactly what Figure 1 showed (and others have previously published) - ie: p-Erk is localized on SF - and that this is also the case if the SF happen to be oriented within the direction of force. No direct evidence of force response is provided, merely a correlation.

Response 11
Stretch-induced changes in actin organization and cell morphology were examined in live cells expressing the F-actin probe, F-Tractin-tdTomato. Sustained uniaxial stretch of blebbistatin-treated cells reinforced actin fibers oriented at small angles against the stretch axis, but not those oriented at large angles (Fig. S9 and Movie 1), which is consistent with the results obtained by observations of fixed cells (Fig. 2A and B). It is noteworthy that actin fibers and cells were not apparently reoriented in response to 5-min (Fig. S9 and Movie 1) or 30-min (data not shown) stretching. Although it has been reported that cyclical stretching of normal fibroblasts or endothelial cells induces reorientation of stress fibers and cells, our experimental condition (sustained stretching of myosin II-inhibited cells) did not lead to the reorientation events. These results strongly suggest that the orientation angle dependencies of F-actin and pERK intensities on actin fibers (Fig. 2A-C) do not come from reorientation of pre-existing fibers. Rather, since the orientation angle is directly related to the magnitude of stretch-generated tensile force on actin fibers, it is conceivable that tensile force-dependent changes in F-actin and pERK intensities would underlie those angle dependencies. These points were mentioned in the main text, Page 5, Line 11-15, and the new data (Fig. S9 and Movie 1) and their figure legends (Supplementary Information, Page 7, Line 16-24, and Page 10, Line 7-12) were added. The method of live cell imaging combined with uniaxial stretching was described in Supplementary Information (Page 4, Line 7-11).

In cells subjected to the stretching, there was a good positive correlation between estimated tensile force and pERK intensity on actin fibers. The tensile force was calculated, based on the mechanical aspect, simply from the stretch percentage, and the obtained force values closely reflect the inputs that we manipulated and applied to cells (i.e., stretch). Therefore, it is conceivable that the positive correlation between tensile force and pERK intensity in Fig. 2E comes from tensile force-dependent changes in pERK intensity. Together with the results that 1) myosin II inhibition-induced decrease in pERK on actin fibers was counteracted by mechanical stretching of myosin II-inhibited cells (Fig. 2), and 2) the positive correlation between tensile force and pERK intensity on individual actin fibers was obtained even in a single stretched cell (Fig. S11), it is very likely that tensile force on each actin fiber is a common factor that leads to increase in pERK on the fiber. Furthermore, we added new data showing that disruption of stress fibers by the treatment with cytochalasin D abolished stretch-induced phosphorylation of ERK, which further supports the notion that tension in stress fibers is critical for facilitating ERK phosphorylation. This new data was shown in Fig. S12, and its figure legend was added in Supplementary Information, Page 8, Line 13-24.

Point 12
Similar concerns relate to figure 3 - these measurements are based on analysis of fixed images of cells and assumptions made about local force production by individual SF.

Response 12
Contractile force generated in stress fibers is a major origin of traction force exerted onto the extracellular substrate (Kumar et al., 2006, Biophys J 90:3762). In contrast to conventional continuum gel-based measurements of traction force, microforce sensor arrays with micro-pillars enable us to measure forces at individual stress fiber-focal adhesion complexes distinctively. Combination of cell fixation and traction force measurement using micro-pillars has been employed (Tan et al., 2003, PNAS 100:1484; Yang et al., 2011, Nat Protoc 6:187). To estimate tensile force in individual stress fibers using micro-pillars, we set the following criteria for the analysis. 1) Pillars connected with multiple stress fibers were excluded from analyses. Some stress fibers have branching or interconnection in their middle parts, which makes it difficult to estimate tensile force in individual stress fibers (Chang and Kumar, 2013, J Cell Sci 126:3021). To exclude the effect of branching or interconnection, 2) we analyzed only regions of stress fibers in the vicinity (5 μm) of their tips on pillars. These criteria ensure to obtain the force-pERK relationship in the region where force in a single fiber is directly transmitted to a single pillar. These criteria were described in Supplementary Information, Page 4, Line 22-25, and depicted in Fig. S19.

Point 13

Response 13

We reduced the size of arrows to avoid interfering with F-actin and pERK images in Fig. 3A.

The aforementioned results without direct measurements of force (Figs. 1, 2 and S11) suggest that tensile force is a plausible key mediator for the myosin II-dependent increase in pERK on stress fibers, as discussed in **Response 11**. A direct measurement of myosin II-dependent tensile force in stress fibers shown in Fig. 3 highly strengthens this notion; the result shows the direct, positive correlation between the pERK intensity and myosin II-based tensile force on individual stress fibers. Since myosin II activity increases both the F-actin amount in individual stress fibers (Fig. 2 and Fig. S6) and the relative amount of pERK against F-actin in the fibers (Fig. 1 and Fig. S1), myosin II-based tensile force would lead to an increase in pERK on stress fibers in a synergistic way. These points were mentioned in the main text, Page 7, Line 14-16.

Point 14

Response 14

As discussed above, our results showed that tensile force in stress fiber plays a role in increasing ERK phosphorylation. Besides classical stress fibers connecting focal adhesions, actomyosin bundles are formed between cell-cell junctions as well. We therefore extended our study to actomyosin bundles connecting cell-cell junctions. To exclude contributions of classical stress fibers, we employed the epithelial cell sheet suspended over the ECM-devoid region; cells in the sheet did not form focal adhesion-stress fiber complexes, and were held together by actomyosin bundles interconnected through cell-cell junctions. The result with the suspended cell sheet showed myosin II-dependent localization of pERK on the actomyosin bundles connecting between cell-cell junctions. This implies that not only classical stress fibers but actomyosin bundles in general play a role in tensile force-induced ERK phosphorylation. We modified the manuscript to clarify these points (Page 7, Line 19-22 in the main text).

Response to the Reviewer 3

We thank the reviewer very much for the helpful comments. We have extensively modified our manuscript according to the comments (modifications are colored in red in the revised manuscript). Below are our point-by-point responses to the Reviewer's comments.

Point 1

Response 1

Thank you for the interesting suggestion. We compared distribution of phosphorylated ERK with those of α-actinin and vinculin. Distribution of pERK on stress fibers tended to be exclusive to that of α-actinin, and pERK did not show apparent localization at vinculin-positive focal adhesions. These points were mentioned in the main text, Page 4, Line 19-21, and the results were shown in Fig. S4.

Point 2

Second I wonder if it can be shown that the whole signaling cascade is active; showing phosphorylated ERK does not prove that the tension has a functional effect on the cells.

Response 2

We examined the phosphorylation status of the major ERK effector, RSK. Stretch-induced ERK phosphorylation on stress fibers was associated with increased phosphorylation of RSK. However, when stress fibers were disrupted upon cytochalasin D treatment, mechanical stretch did not induce phosphorylation of ERK and RSK. This suggests that tension-dependent ERK phosphorylation on stress fibers activates downstream signaling including RSK phosphorylation. These points were mentioned in the main text, Page 6, Line 18-22, and the results were shown in Fig. S12.

Point 3

Third I find that a few references are missing that earlier have provided insight into the relation between tension and protein localization using laser cutting. Apart from Ref. 54 (Kumar et al. Biophysical Journal 2006), Colombelli et al. Journal of Cell Science 2009 and Tanner et al. Biophysical Journal 2010 should be cited and discussed. In general, laser cutting would also be a great tool to address the dynamics of ERK-activation.

Response 3

As the reviewer pointed out, studies with laser cutting of stress fibers have provided important insights on how tension in stress fibers affects cell shape and protein localization at stress fibers as well as focal adhesions. We cited those studies including the one the reviewer indicated. However, those studies have not measured tension or tensile force per se in stress fibers. By contrast, in our present study, we estimated tensile forces acting on individual stress fibers with two different approaches (mechanical modeling of stretched stress fibers and the micro-pillar assay), and thereby showed the quantitative relationship between tensile force and ERK phosphorylation on individual stress fibers. We agree that laser cutting is a potential great tool to address the relationship between tension in the stress fiber and ERK activation. But, it is quite challenging to combine dynamic monitoring of the ERK phosphorylation status with laser cutting of individual stress fibers, which needs to be achieved in future studies. We mentioned these points in the main text, Page 7, Line 4-11.

2nd Editorial Decision   04 December 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.