Frustration of endocytosis potentiates compression-induced receptor signaling

Francesco Baschieri, Dahiana Le Devedec, Samuel Tettarasar, Nadia Elkhatib and Guillaume Montagnac

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Original submission

First decision letter

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MS TITLE: Endocytosis frustration potentiates compression-induced receptor signaling

AUTHORS: Francesco Baschieri, Dahiana Le Devedec, Nadia Elkhatib, and Guillaume Montagnac
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

As you will see, the reviewers share enthusiasm for the concept you seek to establish, of compression induced receptor signaling. However, they each raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They phrase them differently but all are pointed towards major new experiments to clarify: key aspects of the signaling mechanism, the precision of the experiments, and the description of the results in the text. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. I caution you that these reviews read to me as on the borderline of recommending reject / major revisions. I am not convinced that their diverse concerns can be addressed in a normal revision period. I decided to allow revision based on your prior work in this field and the expectation that you can address their experimental concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers. If you find that you need a reasonable extension of the revision period to address their concerns, feel free to reach out.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this work, continuous compressive perturbations are used to explore the impact of mechanically-induced pressure on Clathrin-coat structures (CCS) assembly and dynamics and on CCS-dependent EGFR signaling.
The authors provide some evidence that compression possibly leading to increased membrane tension causes, in turn, the inhibition of CCS dynamics. This also results in the accumulation at CCS of EGFR and elevation of ERK1/2 signaling possibly as consequences of the compression-mediated increase in the shedding of HB-EGF.

The experiments are in general well-conducted, however, they also appeared overinterpreted and insufficient in providing cogent support to the model proposed according to which compression might lead to increased membrane tension (not proven), in turn, capable of promoting EGFR clustering and activation (not proven) at CCS, possibly due to increased shedding of HB-EGF (again not proven).

Comments for the author

Hence, the manuscript while containing some interesting findings, needs to be corroborated by a set of additional, key experimental evidence. Specifically:
1. EGFR is proposed but indirectly shown to be active at CCS. The use of specific anti-phospho EGFR antibody would seem a straight forward way to address this point.
2. Membrane tension is proposed to be a critical determinant in the process. If so it should be directly measured or the concept should be supported by other means and experiments aimed at showing whether increasing membrane tension leads to EGFR recruitment to CCS, the liberation of HB-EGF, and activation of the receptors. To this end, the author could use stretching devices or altered membrane tension by hypotonic stimulation, or by more sophisticated pipette-pulling methodologies.
3. They should also show that compression is sufficient to increase HB-EGF shedding by directly measuring it or measuring the cleaved EGF or by using condition media from compressed and not compressed cells and test whether this is sufficient to elicit EGFR activation.
4. Finally, one additional tenet implied by the reported finding is that in the presence of the appropriate ligand (as in the case of HGF for MET receptors) compression might be sufficient for the recruitment of receptor to CCS. This should be tested using also other ligands for the receptors, such as Angiotensin Receptor-1, and GPCR, that are shown not to be relocalized to CCS in response to compression.
5. Steady-state compression is shown to lead to a transient increase in ERK1/2. What is the cause of this dynamics behavior? Does it correlate with delayed internalization of EGFR? Is it due to the EGFR inactivation?

Is compression also sufficient to cause an ERK1/2-dependent increase in proliferation?
Specific comments

In figure S2E, it is shown that compression leads to exclusion of GFP-YAP1 from the nucleus. This is a bit odd in light of the finding reported by Pere' Roca-Cusack group showing that AFM-mediated compression of cell nuclei triggers, instead, nuclear entry of YAP1. Regardless, it is unclear what is the need of using GFP-YAP1 in this context.

In the Tschumperlin manuscript (nature 2004) the activation of EGFR is not only dependent on HB-EGF shedding but also on the reduction of the intercellular space in HBEC pseudostratified layers. These conditions are completely different from the experimental setting performed in this manuscript where individual HELA cells are subjected to compression. Thus, caution should be used in extending findings obtained the Tschumperlin's manuscript to the system under investigation here.

In figure 3B, it is shown that Batimastat, which according to the authors should prevent HB-EGF shedding and thus activation of the EGFR, prevents EGFR re-localization of CCS. However, EGFR inhibition of its kinase activity failed to do. It is far from obvious how to reconcile these apparently opposite findings. In all these experiments analysis of phosphorylation of the EGFR should be examined.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Baschieri et al. examines the changes in clathrin coated structures (CCSs) and receptor signaling which occur when cultured cells are submitted to a compression force. This force presumably increases membrane tension, which is known to inhibit clathrin mediated endocytosis by inhibiting membrane invagination. The authors show that CCS dynamics are inhibited by compression and that tyrosine kinase receptor signaling is induced. In particular, they show that EGFR are recruited to CCSs independently of an exogenous ligand, but likely comes from paracrine HB-EGF processing from metalloproteases. Furthermore, recruitment of EGFR to CCSs induces signaling assessed by ERK phosphorylation. This ligand-independent signaling depends on the presence of CCS because it is abolished by clathrin or adaptin knock-down. Furthermore, they show that other RTKs, such as hepatocyte growth factor receptors, can be further recruited to CCS and induce ERK phosphorylation after compression. The results are interesting for the mechanistic analysis of a cellular process but also because they are relevant for the activation of this essential signaling pathway in a context of high pressure such as inside tumors, which would warrant publication. I have a few remarks which should be addressed before it is suitable for publication.

Comments for the author

Here are my suggestions:
Major points:
1. One important aspect of the model developed by the authors is that CHC or adaptin KD abolishes CCSs. However, this is not systematic, as shown for example in Pascolutti et al. cell Reports 2019. The authors should use Clc-GFP in AP2-KD cells (or mu2-GFP in CHC KD cells) to test if CCS indeed disappear in these conditions. What is the effect of adaptin KD or CHC KD on CCS (or mu2) dynamics before and after cell compression?
2. The lack of AT1R/bArr/HGFR recruitment to CCS after compression is interesting but the authors must show positive controls that after ligand binding these cargo are indeed recruited to CCSs.
3. The mechanism of the relative decrease in TfR-EGFP enrichment to CCSs (Figure S4) is unclear. It could be either a loss of Tfr targeting to CCS which could be explained by a lack of binding to AP2. Alternatively, it could be due to saturation of AP2 binding sites if endocytosis is inhibited but not exocytosis, providing more TfR to the plasma membrane. The authors could measure the total increase in TfR-EGFP fluorescence during cell compression. If this increases significantly it would be in favor of the second explanation.

Minor points:
1. It would be nice to have a cartoon explaining the experimental procedure for the cell compression. Is the amount of liquid medium before compression precisely controlled?
2. In page 6, why do the authors talk about the effect of membrane tension in yeast? I would either make a general comment in the introduction, or here refer only to work on mammalian cell lines.
3. Please remind the reader what Gefitinib blocks, perhaps with a reference.
4. Page 8: The logic behind the EGFR-GFP FRAP experiment is not very clear. Please explain further.
5. Page 11: Some recent studies (2002 and 2005!). Please remove the word ‘recent’ or cite more recent work.
Typos:
Page9: Remove “that” from “… accumulation at compression-induced frustrated CCSs that.”
Page 10: Add a verb to “We also reported that AP-2 dynamics perturbed at frustrated CCSs under compression.”
Please read again the manuscript carefully, as other typos may have escaped my attention.
Data presentation:
Please show individual points for histograms (all Western blot quantifications)
Methods:
Please include the characteristics of the emission filters for the spinning disk confocal microscope.
TIRF/FRAP system: is the ‘motorized device’ for positioning part of the Nikon Ti2 Eclipse system, or of another manufacturer? Please provide more details.

Reviewer 3

Advance summary and potential significance to field

The article authored by Dr. Guillaume Montagnac and colleagues analyzes the connection between increased membrane tension, endocytosis, and signaling; it shows that frustrated clathrin coated structures (CCS) can be hubs for signaling of both epidermal growth factor receptor and hepatocyte growth factor receptor.
In conclusion the authors propose that CCS frustration can be a "sensor" for the cells to adapt to the surrounding environment. The reviewer thinks that the work is of interest.

Comments for the author

The reviewer has the following comments:
1) Are the cells viable after compression? Can be added an experiment to demonstrate hat the cells can survive this stress? The induction of blebs at the surface and in the nucleous suggests a lethal stimuli.
2) The authors use CRISPR/Cas9 knock-in gene editing to study the assembly of CCS. There is no characterization of the developed gene edited cell lines. It is not clear if single clones were developed and which criteria were used to choose such clones. Since HeLa cells are tetraploids, only a partial editing is most likely achieved, that results in few copies of the protein of interest being labelled. In consequence, low expression of the EGFP or mCherry AP2M1 subunit will not be sufficient to detect short lived and small CCS (see Aguet F. et al., Dev Cell 2013). It would be important to add as supplemental material a figure that address this concern, or refer the reader to previous publications that include this information.
3) The authors state: "We chose to FRAP individual CCSs corresponding to clathrin-coated plaques because the long-lived nature of these structures allows to monitor fluorescence recovery over minutes." TIRF-SIM analysis of non-diffraction limited CCS demonstrated that those are composed of a mixture of flat arrays and forming clathrin coated vesicles (CCVs) (Li D. et al., Science 2015). Therefore the FRAP experiment does not measure the recovery of mobile or immobile fraction of clathrin in plaques, but most likely show the impairment of budding of CCVs that form in these non-diffraction limited objects. In fact, only 20% of the total signal result immobile, suggesting that 80% is consisting of budding CCVs. The authors should include this argument to better inform the audience.
4) The authors do not define clearly what they intend with CCS. Are those the canonical CCVs that assemble in ~60s composed of ~60 triskelia (Herlich et al., 2004)? Are CCS both CCV and plaques? Please clarify the nomenclature to resolve potential ambiguous interpretations. The authors seem to use the word regardless, while they use CCS to define plaques only in their previous work
(e.g. Baschieri et al., 2018). This is a critical issue to unambiguously understand the presented data.
5) In Fig. 2 upon depletion of components of the clathrin machinery the authors observe a very mild decrease in p-ERK activation: the effect is even milder upon the complete KO of CHC (Fig.S2 g) than upon the partial down regulation of AP2 subunits. It is not clear if any dynamics of CCS is still present. How are the AP2 adaptors organized upon pressure? An experiment showing the dynamics of CCS upon depletion AP2 depletion or of AP2 upon CHC depletion can elegantly complete the work.
6) The reviewer finds very interesting the differential distribution of the receptors upon increased pressure. From the experiment in Fig. S4 it seems that only half of the transferrin receptors re-localize from the CCS to the cell surface. The panels in Fig. S4 show a dramatic increase of the TFR fluorescence intensity on the cell surface upon compression of the cell: can the authors add the dynamic movie of this interesting experiment? A potential explanation for this result is that signaling receptors such as EGFR displace TFRs from the CCS binding to the available AP2s. Did the authors considered to perform an experiment expressing both EGFR and TFR? This experiment would be extremely informative because the ratio of the two signals may support the previously mentioned hypothesis.

Minor:

1) In Fig. S2 g the authors probe a membrane for AP2 after depleting either AP2M1 or AP2A1: which is this subunit? From the material and method section appears that only a mouse monoclonal anti-α-adaptin was used in the experiments.

First revision

Author response to reviewers' comments

Reviewer 1

In this work, continuous compressive perturbations are used to explore the impact of mechanically-inducedpressure on Clathrin-coat structures (CCS) assembly and dynamics and on CCS-dependent EGFR signaling. The authors provide some evidence that compression possibly leading to increased membrane tension causes, in turn, the inhibition of CCS dynamics. This also results in the accumulation at CCS of EGFR and elevation of ERK1/2 signaling possibly as consequences of the compression-mediated increase in the shedding of HB-EGF. The experiments are in general well-conducted, however, they also appeared overinterpreted and insufficient in providing cogent support to the model proposed according to which compression might lead to increased membrane tension (not proven), in turn, capable of promoting EGFR clustering and activation (not proven) at CCS, possibly due to increased shedding of HB-EGF (again not proven).

Hence, the manuscript while containing some interesting findings, needs to be corroborated by a set of additional, key experimental evidence.

Specifically:
1. EGFR is proposed but indirectly shown to be active at CCS. The use of specific anti-phospho EGFR antibody would seem a straight forward way to address this point.

Despite our efforts, we were unable to find an anti-phospho EGFR working for immunofluorescence in our hands. To try to address this important issue, we used anti-phosphorylated tyrosines staining as we previously showed that this is a good proxy to detect activated EGFR at CCSs (Baschieri et al, Nat Comm 2018). We observed that anti-P-Tyr is readily detectable at CCSs upon compression and is lost when cells are treated with gefitinib (Fig. S3c and d). Together with the fact that we consistently observe GFP-EGFR being recruited at CCSs under compression, these new experiments strongly suggest that EGFR is active at CCSs in compressed cells.

2. Membrane tension is proposed to be a critical determinant in the process. If so it should be directly measured or the concept should be supported by other means and experiments aimed at showing whether increasing membrane tension leads to EGFR recruitment to CCS, the liberation of HB-EGF, and activation of the receptors. To this end, the author could use stretching devices or altered membrane tension by hypotonic stimulation, or by more sophisticated pipette-pulling methodologies.

Measuring membrane tension in confined cells is very challenging as cells are not easily accessible. However, we followed the reviewer’s advice to increase membrane tension using hypotonic shocks. We now report that EGFR accumulates at CCSs and that ERK gets activated in these conditions (Fig. S2a and b and Fig. S3a and b) thus mirroring the results we obtained with compression.

3. They should also show that compression is sufficient to increase HB-EGF shedding by directly measuring it or measuring the cleaved EGF or by using condition media from compressed and not compressed cells and test whether this is sufficient to elicit EGFR activation.

Following the advice of the reviewer, we stimulated control cells with conditioned medium from compressed cells. We indeed observed that this was sufficient to activate ERK as shown in Fig. S3e and f.
In addition, we observed that this effect was lost if using conditioned medium from cells compressed in the presence of batimastat (Fig. S3e and f). These data suggest that the previously published mechanism whereby a pro-EGFR ligand is cleaved and released upon compression and leads to EGFR activation holds true in our experimental setup (Tschumperling 2004).

4. Finally, one additional tenet implied by the reported finding is that in the presence of the appropriate ligand (as in the case of HGF for MET receptors) compression might be sufficient for the recruitment of receptor to CCS. This should be tested using also other ligands for the receptors, such as Angiotensin Receptor-1, and GPCR, that are shown not to be relocalized to CCS in response to compression.

We now provide experiments showing that stimulation using a suboptimal concentration of angiotensin II leads to AT1R recruitment at CCSs in compressed but not in uncompressed cells (Fig. 5c and d).

5. Steady-state compression is shown to lead to a transient increase in ERK1/2. What is the cause of this dynamics behavior? Does it correlate with delayed internalization of EGFR? Is it due to the EGFR inactivation? Is compression also sufficient to cause an ERK1/2-dependent increase in proliferation?

In the literature, ERK activation is consistently reported to be transient upon specific receptor stimulation. The reviewer is right that endocytosis is proposed to participate in signaling shutdown and thus in the transient nature of signal activity. Yet, many receptors, including EGFR continue to signal upon endocytosis and the kinetics of EGFR-dependent signaling and EGFR endocytosis and degradation do not necessarily match (Sorkin and von zastrow, Nat Rev Mol Cell Biol 2009). In addition, it should be noted that CCS dynamics is reduced but not completely stalled in compressed cells (Fig. 1b), opening the possibility that endocytosis still occurs, although most likely at a lower rate. It is thus very difficult to address the reviewers question as many mechanisms could be involved such as for example activation of receptor phosphatases at the cell surface. We thus believe addressing in depth the mechanisms regulating ERK transient activation are beyond the scope of the present manuscript.

We also now report that compression does not significantly affect cell death or cell proliferation (Fig. S1f and g).

Specific comments
In figure S2E, it is shown that compression leads to exclusion of GFP-YAP1 from the nucleus. This is a bit odd in light of the finding reported by Pere' Roca-Cusack group showing that AFM-mediated compression of cell nuclei triggers, instead, nuclear entry of YAP1. Regardless, it is unclear what is the need of using GFP-YAP1 in this context.

Because YAP is considered to be a central actor in mechanotransduction pathways, we initially used GFP-YAP as a control. It came to our surprise that GFP-YAP did not accumulate in the nucleus upon cellular compression while GFP-ERK did. As compared to the Roca-Cusack's paper, the difference may come from the level of forces used to compress the cell/nucleus. We estimated that we apply a force that is 3 orders of magnitude higher as compared to the Roca-Cusack paper (1 kPa = $1*10^{-3}$ N/mm^2 in our assays while a force of $2.4*10^{-6}$ N/mm^2 was used in the case of the Roca-Cusack paper). The difference may also stem from the direct and specific squeezing of the nucleus using AFM in the Roca-Cusack story while we applied forces on the whole cell.
Our experimental setup may relate more directly to situations in which cells are highly confluent/confined and in which YAP was shown to exit the nucleus (Dupont et al, Nature 2011).

In the Tschumperlin manuscript (nature 2004) the activation of EGFR is not only dependent on HB-EGF shedding but also on the reduction of the intercellular space in HBEC pseudostratified layers. These conditions are completely different from the experimental setting performed in this manuscript where individual HELA cells are subjected to compression. Thus, caution should be

used in extending findings obtained the Tschumperlin's manuscript to the system under investigation here.

The referee is right. It could be argued that the agarose gel used to compress cells in our assays may, to some extent, "concentrate" the ligand around cells. Yet, our new experiments showing that conditioned medium from compressed cells can induce ERK activation in naïve, uncompressed cells rather suggest that the simple presence of the ligand is sufficient to obtain the measured Erk activation, independently of confinement, at least in our experimental setup (Fig. S3e and f).

In figure 3B, it is shown that Batimastat, which according to the authors should prevent HB-EGF shedding and thus activation of the EGFR, prevents EGFR re-localization of CCS. However, EGFR inhibition of its kinase activity failed to do. It is far from obvious how to reconcile these apparently opposite findings. In all these experiments analysis of phosphorylation of the EGFR should be examined.

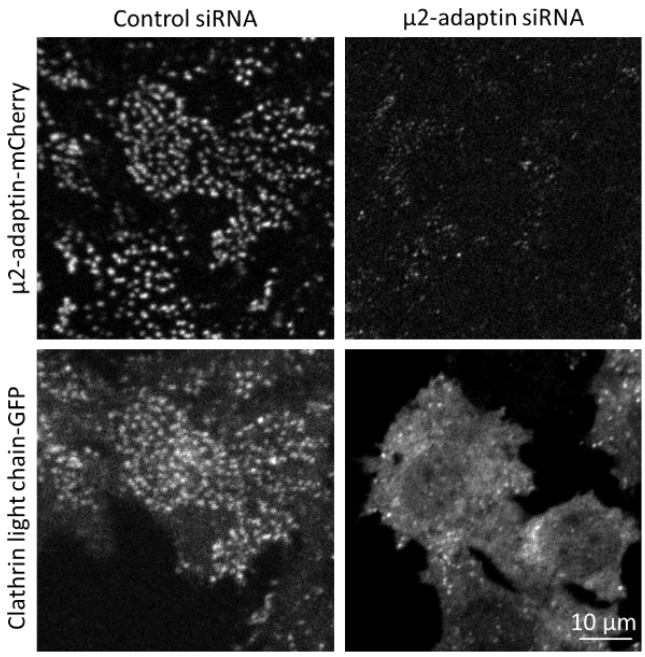
It is true that it is classically assumed that EGFR needs first to be activated before to be recruited to CCSs. However, the first step of activation is EGFR dimerization and this step alone was reported to be sufficient for EGFR to accumulate at CCSs, independently of EGFR kinase domain trans-phosphorylation/activation (Wang et al, Mol Cell Biol 2002; Wang et al, EMBO Rep 2005). Our data are in agreement with this model as stated in the manuscript: HB-EGF is required (presumably leading to EGFR dimerization) but EGFR kinase domain activity is not required for recruitment at CCSs.

Reviewer 2

The manuscript by Baschieri et al. examines the changes in clathrin coated structures (CCSs) and receptor signaling which occur when cultured cells are submitted to a compression force. This force presumably increases membrane tension, which is known to inhibit clathrin mediated endocytosis by inhibiting membrane invagination. The authors show that CCS dynamics are inhibited by compression and that tyrosine kinase receptor signaling is induced. In particular, they show that EGFR are recruited to CCSs independently of an exogenous ligand, but likely comes from paracrine HB-EGF processing from metalloproteases. Furthermore, recruitment of EGFR to CCSs induces signaling assessed by ERK phosphorylation. This ligand-independent signaling depends on the presence of CCS because it is abolished by clathrin or adaptin knock-down. Furthermore, they show that other RTKs, such as hepatocyte growth factor receptors, can be further recruited to CCS and induce ERK phosphorylation after compression. The results are interesting for the mechanistic analysis of a cellular process but also because they are relevant for the activation of this essential signaling pathway in a context of high pressure such as inside tumors, which would warrant publication. I have a few remarks which should be addressed before it is suitable for publication.

Here are my suggestions:
Major points:
1. One important aspect of the model developed by the authors is that CHC or adaptin KD abolishes CCSs. However, this is not systematic, as shown for example in Pascolutti et al. cell Reports 2019. The authors should use Clc-GFP in AP2-KD cells (or mu2-GFP in CHC KD cells) to test if CCS indeed disappear in these conditions. What is the effect of adaptin KD or CHC KD on CCS (or mu2) dynamics before and after cell compression?

We previously published that AP-2 depletion leads to a drastic ablation of most CCSs in HeLa cells (Montagnac et al Curr Biol 2011). We repeated these experiments and present them below:



However, given that we already published such results and that AP-2 is well accepted to be a central hub required for the formation of most CCSs in physiological conditions (McMahon and Boucrot, NRMCB 2011; Metlen et al, Annu Rev Biochem 2018), we chose not to incorporate them into the manuscript.
Because there is almost no more CCSs in AP-2-depleted cells, and because CHC is absolutely required for CCSs to bud, we believe that, in the scope of the present manuscript, it is not relevant to test the behavior of such non-physiological structures under confinement.

2. The lack of AT1R/bArr/HGFR recruitment to CCS after compression is interesting but the authors must show positive controls that after ligand binding these cargo are indeed recruited to CCSs.

The referee raises a very valid point and data showing that AT1R and HGFR constructs are functional (meaning that they can be recruited at CCSs upon stimulation) are now included in the manuscript (Fig. S5b and c).

3. The mechanism of the relative decrease in TfR-EGFP enrichment to CCSs (Figure S4) is unclear. It could be either a loss of Tfr targeting to CCS which could be explained by a lack of binding to AP2. Alternatively, it could be due to saturation of AP2 binding sites if endocytosis is inhibited but not exocytosis, providing more TfR to the plasma membrane. The authors could measure the total increase in TfR-EGFP fluorescence during cell compression. If this increases significantly it would be in favor of the second explanation.

Following the referee's advice, we indeed measured an increase of the plasma membrane level of TfR upon compression (Fig. 4c). We believe, as suggested by the referee, that this most likely reflects the lack of TfR endocytosis (that is usually very efficiently internalized) and this is now discussed in the manuscript.

Minor points:
1. It would be nice to have a cartoon explaining the experimental procedure for the cell compression. Is the amount of liquid medium before compression precisely controlled?

We now provide a schematic of the setup in Fig S1e.

2. In page 6, why do the authors talk about the effect of membrane tension in yeast? I would either make a general comment in the introduction, or here refer only to work on mammalian cell lines.

We now refer to a work in mammalian cells.

3. Please remind the reader what Gefitinib blocks, perhaps with a reference.

Done

4. Page 8: The logic behind the EGFR-GFP FRAP experiment is not very clear. Please explain further.

We modified figures and text to make this point clearer

5. Page 11: Some recent studies (2002 and 2005!). Please remove the word 'recent' or cite more recent work.

Done

Typos:
Page9: Remove "that" from "… accumulation at compression-induced frustrated CCSs that."
Page 10: Add a verb to "We also reported that AP-2 dynamics perturbed at frustrated CCSs under compression."
Please read again the manuscript carefully, as other typos may have escaped my attention.
All done.

Data presentation:
Please show individual points for histograms (all Western blot quantifications)

Done.

Methods:
Please include the characteristics of the emission filters for the spinning disk confocal microscope.

Done

TIRF/FRAP system: is the 'motorized device' for positioning part of the Nikon Ti2 Eclipse system, or of another manufacturer? Please provide more details.

Done

Reviewer 3
The article authored by Dr. Guillaume Montagnac and colleagues analyzes the connection between increased membrane tension, endocytosis, and signaling; it shows that frustrated clathrin coated structures (CCS) can be hubs for signaling of both epidermal growth factor receptor and hepatocyte growth factor receptor.
In conclusion the authors propose that CCS frustration can be a "sensor" for the cells to adapt to the surrounding environment. The reviewer thinks that the work is of interest.

The reviewer has the following comments:

1) Are the cells viable after compression? Can be added an experiment to demonstrate that the cells can survive this stress? The induction of blebs at the surface and in the nucleous suggests a lethal stimuli.

We now provide data showing that neither cell death nor cell proliferation are significantly affected by the 30 min compression period (Fig. S1f and g).

2) The authors use CRISPR/Cas9 knock-in gene editing to study the assembly of CCS. There is no characterization of the developed gene edited cell lines. It is not clear if single clones were developed and which criteria were used to choose such clones. Since HeLa cells are tetraploids,

only a partial editing is most likely achieved, that results in few copies of the protein of interest being labelled. In consequence, low expression of the EGFP or mCherry AP2M1 subunit will not be sufficient to detect short lived and small CCS (see Aguet F. et al., Dev Cell 2013). It would be important to add as supplemental material a figure that address this concern, or refer the reader to previous publications that include this information.

These cell lines have been generated and used in a previous publication (Baschieri et al, Nat Comm 2018). We now provide data showing that virtually all u2-mCherry (or mu2-GFP) positive structures are also positive for endogenous a-adaptin (Fig S1a).

3) The authors state: “We chose to FRAP individual CCSs corresponding to clathrin-coated plaques because the long-lived nature of these structures allows to monitor fluorescence recovery over minutes.” TIRF-SIM analysis of non-diffraction limited CCS demonstrated that those are composed of a mixture of flat arrays and forming clathrin coated vesicles (CCVs) (Li D. et al., Science 2015). Therefore the FRAP experiment does not measure the recovery of mobile or immobile fraction of clathrin in plaques, but most likely show the impairment of budding of CCVs that form in these non-diffraction limited objects. In fact, only 20% of the total signal result immobile, suggesting that 80% is consisting of budding CCVs. The authors should include this argument to better inform the audience.

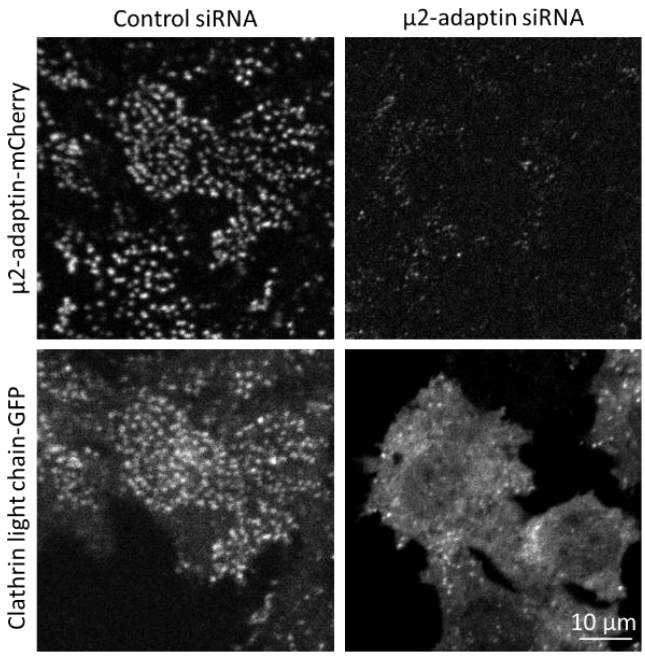
We respectfully disagree with the referee. The kinetics of fluorescence recovery in our FRAP assays (t1/2≈8s) is way too fast to reflect vesicular budding as a major cause of fluorescence turnover. Indeed, it is generally admitted that budding CCSs lifetime is between 30s and 60s depending on cell types. We also previously published that clathrin light chain turnover at CCSs shows an even faster kinetics (t1/2≈5s; FRAP assays performed in the same conditions and cell type as the present study; Montagnac et al, Curr Biol 2011). In addition, it was shown that FRAP values of clathrin light chain are not affected by endocytosis inhibition (Wu et al J Cell Biol 2001). Thus, the kinetics of fluorescence recovery mostly reflects protein turnover at CCSs, rather than vesicular budding.

4) The authors do not define clearly what they intend with CCS. Are those the canonical CCVs that assemble in ~60s composed of ~60 triskelia (Herlich et al., 2004)? Are CCS both CCV and plaques? Please clarify the nomenclature to resolve potential ambiguous interpretations. The authors seem to use the word regardless, while they use CCS to define plaques only in their previous work (e.g. Baschieri et al., 2018). This is a critical issue to unambiguously understand the presented data.

We use CCS as a generic term to refer to all clathrin-coated structures present at the plasma membrane. We now added this sentence in the main text to clarify this issue: “HeLa cells display a mixture of dynamic, diffraction limited CCSs, corresponding to canonical clathrin-coated pits, and long-lived, large CCSs, corresponding to clathrin-coated plaques.”

5) In Fig. 2 upon depletion of components of the clathrin machinery the authors observe a very mild decrease in p-ERK activation: the effect is even milder upon the complete KO of CHC (Fig.S2 g) than upon the partial down regulation of AP2 subunits. It is not clear if any dynamics of CCS is still present. How are the AP2 adaptors organized upon pressure? An experiment showing the dynamics of CCS upon depletion AP2 depletion or of AP2 upon CHC depletion can elegantly complete the work.

We previously published that AP-2 depletion leads to a drastic ablation of most CCSs in HeLa cells (Montagnac et al Curr Biol 2011). We repeated these experiments and present them below:



However, given that we already published such results and that AP-2 is well accepted to be a central hub required for the formation of most CCSs in physiological conditions (McMahon and Boucrot, NRMCB 2011; Metlen et al, Annu Rev Biochem 2018), we chose not to incorporate them into the manuscript.
Because there is almost no more CCSs in AP-2 depleted cells, and because CHC is absolutely required for CCSs to bud, we believe that, in the scope of the present manuscript, it is not relevant to test the behavior of such non-physiological structures under confinement.

We should also mention that the decrease in p-Erk upon clathrin machinery depletion is not so mild as it is more than twice reduced as compared to the control. Yet, the referee raises an interesting point regarding the differential impact of AP-2 versus clathrin depletion. As shown above, in the absence of AP-2, the number of CCSs is drastically reduced. However, we previously published that in the absence of clathrin, although we cannot talk about CCSs anymore, a core structure composed of AP2 and other adaptors and still able to recruit receptors, can still be found at the plasma membrane (Elkhatib et al, Science 2017; Baschieri et al, Nat Comm 2018). We even provided an ultrastructural characterization of such clathrin-less structures (Elkhatib et al, Science 2017). Thus, it is indeed possible that the absence of structure (AP-2 depletion) has deeper consequences than a disorganization of these structures (clathrin-depletion). However, we chose not to discuss these issues in the present manuscript because the difference between these two conditions is not statistically significant.

6) The reviewer finds very interesting the differential distribution of the receptors upon increased pressure. From the experiment in Fig. S4 it seems that only half of the transferrin receptors re-localize from the CCS to the cell surface. The panels in Fig. S4 show a dramatic increase of the TFR fluorescence intensity on the cell surface upon compression of the cell: can the authors add the dynamic movie of this interesting experiment? A potential explanation for this result is that signaling receptors such as EGFR displace TFRs from the CCS binding to the available AP2s. Did the authors considered to perform an experiment expressing both EGFR and TFR? This experiment would be extremely informative because the ratio of the two signals may support the previously mentioned hypothesis.

We now added a video (video 3) to document this observation. In addition, following the referee's advice, we investigated the possibility that a competition exists between TfR and EFGR. However, we observed that the TfR was still excluded from CCSs in compressed cells, even in the absence of the EGFR. Thus, we can only conclude that there is no competition between the TfR and the EGFR, in agreement with the literature (Lamaze et al, Mol Biol Cell 1993; Warren et al, JBC 1997). Yet, it remains true that the mechanisms leading to TfR exclusion from CCSs under compression are unclear but we believe that investigating them is beyond the scope of the present manuscript.

Minor:
1)In Fig. S2 g the authors probe a membrane for AP2 after depleting either AP2M1 or AP2A1: which is this subunit? From the material and method section appears that only a mouse monoclonal anti-α-adaptin was used in the experiments.

The reviewer is right that we probed the membrane for α-adaptin after depleting for either AP2M1 or AP2A1. It is well-documented that removal of one component of the AP2 complex causes the loss of all the other components of the protein complex (Motley et al. 2006 - 10.1091/mbc.E06-05-0452 - Boucrot et al. 2010 - 10.1371/journal.pone.0010597).

Second decision letter

MS ID#: JOCES/2019/239681

MS TITLE: Endocytosis frustration potentiates compression-induced receptor signaling

AUTHORS: Francesco Baschieri, Dahiana Le Devedec, Samuel Tettarasar, Nadia Elkhatib, and Guillaume Montagnac
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I think that these can largely be addressed through revisions to the text, in particular in some cases by acknowledging limitations of the data or alternative interpretations that cannot be absolutely ruled out. There is also one part of the data that may be better to exclude and one issue (see Rev 3) that would benefit from Western blotting. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors are to be commended as they performed a number of experiments in order to address the points and shortcomings raised. The revised manuscript is significantly improved.

Comments for the author

Few points remain that can be addressed by changes in the text 1. Specific comments Figure S2E, shows that compression leads to exclusion of GFP-YAP1 from the nucleus.

Given the inability to reconcile experimental data with some of the previous literature and the relative tangential importance of this set of experiments I would suggest removing this piece of data. The explanation provided below are plausible but not demonstrated cogently and would require a direct side-by-side comparison with AFM mediated nuclear compression clearly outside the scope of the present manuscript

2. Reviewer:In the Tschumperlin manuscript (nature 2004) the activation of EGFR is not only dependent on HB-EGF shedding but also on the reduction of the intercellular space in HBEC pseudostratified layers. These conditions are completely different from the experimental setting performed in this manuscript where individual HELA cells are subjected to compression. Thus, caution should be used in extending findings obtained the Tschumperlin's manuscript to the system under investigation here.
Authors:The referee is right. It could be argued that the agarose gel used to compress cells in our assays may, to some extent, "concentrate" the ligand around cells. Yet, our new experiments showing that conditioned medium from compressed cells can induce ERK activation in naïve, uncompressed cells rather suggest that the simple presence of the ligand is sufficient to obtain the measured Erk activation, independently of confinement, at least in our experimental setup (Fig. S3e and f).
Reviewer: This is again a plausible explanation but it has no experimental support. I would suggest to explicitly underlying the difference of the current set up with respect to the pseudostratified monolayers used In the Tschumperlin manuscript (nature 2004).

Reviewer 2

Advance summary and potential significance to field

Same as for the first submission. The revised manuacript certainly supports the conclusions better.

Comments for the author

The authors have answered appropriately to all my queries. Overall, I would like to congratulate the authors for the quality of their revision and the constructive answers to the reviewers.
For the description of the emission filters, write Semrock instead of SemSemrock (line 3 page 18)
For clarity, I suggest to add in Figure 5 that the cells are pretreated with suboptimal concentrations of ligand, either in the Figure title or on the figure itself.
Along the comments of reviewer 1, I suggest to cite Dupont et al. 2011 for the absence of nuclear localization of Yap-GFP

Reviewer 3

Advance summary and potential significance to field

The article authored by Dr. Guillaume Montagnac and colleagues analyzes the connection between increased membrane tension, endocytosis, and signaling; it shows that frustrated clathrin coated structures (CCS) can be hubs for signaling of both epidermal growth factor receptor and hepatocyte growth factor receptor.
In conclusion the authors propose that CCS frustration can be a "sensor" for the cells to adapt to the surrounding environment. The reviewer thinks that the work is of interest.

Comments for the author

The authors addressed several of the reviewer's comments. Nevertheless there are few points that need some further clarification.
1) Characterization of the AP2M1-EGFP or AP2M1-mcherry cell lines. The authors added a nice panel in Fig. S1 showing that AP2M2-mcherry are positive also for endogenous AP2A1. This panel does not answer the reviewer concern raised at point 2 in the previous revision. The cell lines have been so far poorly characterized because there is no published information available that define how many copies of AP2M1 present in the gene edited cells are actually carrying the fluorescent

tag. To answer this question is sufficient a western blot showing the fraction of AP2M1-EGFP or mcherry in comparison to the AP2M1 wild type subunit. This information is relevant because HeLa cells are tetraploids and therefore there is a high chance that the cellular model used by the authors have a low substitution (few labelled AP2 molecules). This can result in underestimation of CCS lifetime (as previously mentioned, see Aguet F. et al., Dev Cell 2013). The reviewer have never doubted that AP2M1-EGFP or mcherry subunits are correctly incorporated into the AP2 complexes.
2) FRAP experiments. The authors disagree with the referee observation at point 3 of the previous exchange suggesting that 8 sec of coat lifetime is not sufficient to justify the increase in intensity observed by the authors.
The reviewer respectfully disagree with the authors as well for the following reason: assuming a coat life time of ~40s, in 8 second 1/5 of the total intensity is recovered. Since the authors are looking at non-diffraction limited objects it is not possible to exclude that a mixture of growing coats contributes to the recovered intensity, each one at different stages of their growth (again, look at: Li D. et al., Science 2015). In addition, the ambiguity and the impossibility of a clear interpretation of FRAP experiments is at the basis of the recent work of Chen et al., JCB 2019 cited in the discussion by the authors (read Chen et al., 2019 page 3202). Therefore the authors can claim a decrease of fluorescence recovery upon increased compression but cannot attribute it unambiguously to a decreased molecule exchange since their data do not support it. It instead can be due to a slower polymerization of the coat. This again should be added as clarification for the readers.
3) Can the authors explicitly write in the figure S2G legend or in the blot that the AP2 subunit probed is AP2A1? Although molecular weight is indicated it would make easier for the reader to understand it. In addition please explain in the methods that upon knock down of a subunit of AP2 also the others are degraded and therefore that is sufficient to probe for AP2A1 and cite Motley et al. 2006 - 10.1091/mbc.E06-05-0452 and Boucrot et al. 2010
- 10.1371/journal.pone.0010597. It would be fair to explain clearly the methods and approaches to the readers.

Second revision

Author response to reviewers' comments

Reviewer 1
Advance Summary and Potential Significance to Field:
The authors are to be commended as they performed a number of experiments in order to address the points and shortcomings raised. The revised manuscript is significantly improved.

Few points remain that can be addressed by changes in the text
1. Specific comments
Figure S2E, shows that compression leads to exclusion of GFP-YAP1 from the nucleus.
Given the inability to reconcile experimental data with some of the previous literature and the relative tangential importance of this set of experiments I would suggest removing this piece of data. The explanation provided below are plausible but not demonstrated cogently and would require a direct side-by-side comparison with AFM mediated nuclear compression clearly outside the scope of the present manuscript.

The data on YAP has been removed.

2. Reviewer: In the Tschumperlin manuscript (nature 2004) the activation of EGFR is not only dependent on HB-EGF shedding but also on the reduction of the intercellular space in HBEC pseudostratified layers. These conditions are completely different from the experimental setting performed in this manuscript where individual HELA cells are subjected to compression. Thus, caution should be used in extending findings obtained the Tschumperlin's manuscript to the system under investigation here.

Authors: The referee is right. It could be argued that the agarose gel used to compress cells in our assays may, to some extent, “concentrate” the ligand around cells. Yet, our new experiments showing that conditioned medium from compressed cells can induce ERK activation in naïve, uncompressed cells rather suggest that the simple presence of the ligand is sufficient to obtain the measured Erk activation, independently of confinement, at least in our experimental setup (Fig. S3e and f).

Reviewer: This is again a plausible explanation but it has no experimental support. I would suggest to explicitly underlying the difference of the current set up with respect to the pseudostratified monolayers used In the Tschumperlin manuscript (nature 2004).

We clarified this point in the discussion.

Reviewer 2
Advance Summary and Potential Significance to Field:
Same as for the first submission. The revised manuacript certainly supports the conclusions better.

The authors have answered appropriately to all my queries. Overall, I would like to congratulate the authors for the quality of their revision and the constructive answers to the reviewers.
For the description of the emission filters, write Semrock instead of SemSemrock (line 3 page 18)

Done

For clarity, I suggest to add in Figure 5 that the cells are pretreated with suboptimal concentrations of ligand, either in the Figure title or on the figure itself.

Done

Along the comments of reviewer 1, I suggest to cite Dupont et al. 2011 for the absence of nuclear localization of Yap-GFP

Following the advice of reviewer 1, we decided to remove data related to YAP.

Reviewer 3
Advance Summary and Potential Significance to Field:
The article authored by Dr. Guillaume Montagnac and colleagues analyzes the connection between increased membrane tension, endocytosis, and signaling; it shows that frustrated clathrin coated structures (CCS) can be hubs for signaling of both epidermal growth factor receptor and hepatocyte growth factor receptor.
In conclusion the authors propose that CCS frustration can be a “sensor” for the cells to adapt to the surrounding environment. The reviewer thinks that the work is of interest.

The authors addressed several of the reviewer’s comments. Nevertheless there are few points that need some further clarification.

1) Characterization of the AP2M1-EGFP or AP2M1-mcherry cell lines. The authors added a nice panel in Fig. S1 showing that AP2M2-mcherry are positive also for endogenous AP2A1. This panel does not answer the reviewer concern raised at point 2 in the previous revision. The cell lines have been so far poorly characterized because there is no published information available that define how many copies of AP2M1 present in the gene edited cells are actually carrying the fluorescent tag. To answer this question is sufficient a western blot showing the fraction of AP2M1-EGFP or mcherry in comparison to the AP2M1 wild type subunit. This information is relevant because HeLa cells are tetraploids and therefore there is a high chance that the cellular model used by the authors have a low substitution (few labelled AP2 molecules). This can result in underestimation of CCS lifetime (as previously mentioned, see Aguet
F. et al., Dev Cell 2013). The reviewer have never doubted that AP2M1-EGFP or mcherry subunits are correctly incorporated into the AP2 complexes.

We now provide a western blot (Fig S1c) showing that only a fraction of expressed AP2M1 subunits are tagged with GFP or mCherry, as expected.

2) FRAP experiments. The authors disagree with the referee observation at point 3 of the previous exchange suggesting that 8 sec of coat lifetime is not sufficient to justify the increase in intensity observed by the authors. The reviewer respectfully disagree with the authors as well for the following reason: assuming a coat life time of ~40s, in 8 second 1/5 of the total intensity is recovered. Since the authors are looking at non-diffraction limited objects it is not possible to exclude that a mixture of growing coats
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The reviewer's hypothesis has been taken into account and the conclusion derived from these experiments has been accordingly tune down in the main text.

3) Can the authors explicitly write in the figure S2G legend or in the blot that the AP2 subunit probed is AP2A1? Although molecular weight is indicated it would make easier for the reader to understand it. In addition, please explain in the methods that upon knock down of a subunit of AP2 also the others are degraded and therefore that is sufficient to probe for AP2A1, and cite Motley et al. 2006 - 10.1091/mbc.E06-05-0452 and Boucrot et al. 2010 - 10.1371/journal.pone.0010597. It would be fair to explain clearly the methods and approaches to the readers.

Done (see new Fig S2e and Material and methods section).

Third decision letter

MS ID#: JOCES/2019/239681

MS TITLE: Endocytosis frustration potentiates compression-induced receptor signaling

AUTHORS: Francesco Baschieri, Dahiana Le Devedec, Samuel Tettarasar, Nadia Elkhatib, and Guillaume Montagnac
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors clarify the remaining minor concerns

Comments for the author

No further issues to remark

Reviewer 2

Advance summary and potential significance to field

Same as for the first and second submission.

Comments for the author

The authors have answered all my queries and the manuscript is for me acceptable for publication.

Reviewer 3

Advance summary and potential significance to field

The article authored by Dr. Guillaume Montagnac and colleagues analyzes the connection between increased membrane tension, endocytosis, and signaling; it shows that frustrated clathrin coated structures (CCS) can be hubs for signaling of both epidermal growth factor receptor and hepatocyte growth factor receptor.
In conclusion the authors propose that CCS frustration can be a “sensor” for the cells to adapt to the surrounding environment. The reviewer thinks that the work is of interest.

Comments for the author

The authors addressed the concerns raised by the reviewer. This version of the manuscript is strongly improved. Congratulations.