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MRTF potentiates TEAD-YAP transcriptional activity causing metastasis

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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.)

1st Editorial Decision 02 August 2016

Thank you for the submission of your manuscript entitled 'MRTF potentiates TEAD-YAP transcriptional activity for cancer metastasis' (EMBOJ-2016-95137) to The EMBO Journal. Your study has been sent to three referees, and we have received reports from all of them, which I copy below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of major concerns that would need to be addressed before they can support publication of your manuscript in The EMBO Journal. In particular, referee #1 feels your claims on the importance of YAP - MRTF interaction for metastasis are not sufficiently well supported by your data and asks you to provide independent evidence to corroborate these findings (see ref #1, pt. 1,5). This referee also points out the need for you to revise the relevance of your findings in the light of previous literature (ref #1, standfirst and pt. 2) and further consolidate and explore the mechanistic links between MRTF, NocA3 and TEAD signaling by additional analyses (ref #1, pt.6). Both referees #2 and #3 agree in that the link between mechanical stimuli, YAP and MRTF should be explored further (ref #2, pt. 3; ref #3, additional cross-comment). In addition, all referees list technical issues and controls, which would need to be addressed for publication at The EMBO Journal. I judge the comments of the referees to be generally reasonable, thus we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments.

REFEREE REPORTS

Referee #1:

In this report, the authors investigated the physical and functional interaction between MRTF and YAP. Both MRTF and YAP are transcription co-activators and similarly regulated by serum as well as actin cytoskeleton. The authors showed that MRTF and YAP physically associate with each other and positively contribute to YAP target gene expression. MRTF recruits NcoA3 to enhance YAP transcription activity. Reconstitution of the MRTF knockdown 4T1 cells with wild type MRTF rescued tumor metastasis while expression of a YAP binding defective MRTF mutant was not effective. The authors conclude that the MRTF and YAP interaction plays a role in YAP target gene expression and in response to mechanotransduction. It is also concluded that MRTF potentiates YAP transcription activity to stimulate tumor metastasis.

This study has nicely demonstrated a functional interaction between MRTF and YAP, particularly on the recruiting of NocA3 in YAP dependent transcription activation. In addition, it also provides possible explanation about the LATS independent YAP target gene regulation in response to reorganization of actin. The implication of MRTF and YAP interaction in 4T1 tumor metastasis is potentially interesting. However, it should be noted that recent studies have shown that MRTF can functionally and physically interact with YAP and TAZ (Yu et al 2015; Speight et al 2016).

Specific comments

1) In order to conclude that "MRTF potentiates YAP-TEAD transcriptional activity for cancer metastasis", the authors need to show that YAP is critical for lung metastasis of 4T1 cells. The PPXY motif in MRTF may bind to other WW domain proteins important for metastasis. It needs to show that YAP WW domain mutant cannot support metastasis of 4T1, and regaining of MRTF interaction via a second site compensatory mutation restores the tumor metastasis. Without such data, the title is overstated and should be modified.

2) The second sentence in the abstract "It is unclear, however, whether there is any crosstalk between these two proteins (MRTF and YAP)" is inaccurate. The Yu 2015 and Speight 2016 papers clearly examined the interaction between MRTF and YAP/TAZ. Actually, it has been shown that YAP and MRTF cooperate in gene expression and the TAZ WW domain is responsible for MRTF interaction.

3) Fig.3.D. The data is a bit strange. YAP pulldown followed by MRTF IP retains little TEAD whereas YAP pulldown followed by TEAD IP retains significant MRTF. Do the authors have any explanation?

4) Fig.5. The effect of NcoA3 knockdown on endogenous YAP target gene, such as CTGF and Cyr61, should be determined to demonstrate that NcoA3 affects YAP transcription activity. Does MRFT knockdown affect the interaction between YAP and NcoA3?

5) Fig.6A. The interaction between YAP and MTRF is not convincing. Can the authors repeat the experiments with each treatment having independent duplicated samples?

6) Fig.7C. Lat. B treatment causes actin depolymerization. Based on this data, it is not sufficient to conclude that MRTF-YAP mediates the mechanotransduction signal. In order to make such a conclusion, more mechanical stress conditions should be included in the experiments.

Referee #2:

Kim et al report a functional interaction between YAP and MRTF. They demonstrate direct binding between YAP and MRTF, identify the interaction motifs, and test the functional importance of this interaction on regulation of transcription by YAP using in vitro luciferase reporter assays. Using different versions of MRTF that interact with YAP but not with its canonical binding partner SRF and vice versa, they show that the interaction between MRTF and YAP is essential for the regulation of YAP activity.

This is a thorough study with many innovative and well-performed experiments. The conclusions follow from the data and the finding that YAP activity depends on MRTF is highly interesting for the fields of mechanobiology and YAP/Hippo signaling. I only have a couple of suggestions to further test the contribution of SRF:

1. The authors use a truncated ctgf-Luc reporter as a reporter that depends on Yap-TEAD but not MRTF-SRF. In addition to this, the authors should use the 8xGTIIC reporter that contains only TEAD binding sites. This experiment is required to exclude binding by an SRF-MRTF complex to reporter constructs.

2. In addition, the authors should repeat these experiments in an SRF mutant cell line (generated by CRISPR). Given the prominence of the SRF-MRTF complex in our current model of MRTF function, these experiments will be able to rule out/in SRF as a mediator of the MRTF effects on TEAD reporters.

3. The authors argue that MRTF mediates the mechanical regulation of YAP. However, ECM stiffness for example has a major effect on Yap localization. How is MRTF supposed to be the mediator of the mechanical regulation of Yap when it does not affect the same phenotypes? I suggest that the authors assay the localization of Yap under soft/stiff or sparse/dense conditions in cells with and without MRTF activity. This will determine whether the mechanical regulation of Yap requires MRTF.

Referee #3:

The ability of cells to integrate and respond to a plethora of extracellular and intracellular information relies on transcription factors, which translate this information into precise cellular responses. YAP/TAZ are transcriptional coactivators in the Hippo pathway that shuttle between the cytoplasm and the nucleus, where they interact with other transcription factors - most notably TEA domain family members (TEAD) - to bind cis-regulatory elements. Mechanical cues represent a central mode for YAP/TAZ activity control. YAP and TAZ are directly regulated by ECM stiffness, cell shape, and cytoskeletal tension. SRF transcriptional coactivators of the Myocardin-related transcription factor (MRTF) family also respond to changes in the actin cytoskeleton and RhoA activation and previous studies revealed a crosstalk between YAP and MRTF. This functional relationship includes the activation of TAZ transcription by SRF-MRTF and the interaction of MRTF-A and YAP to promote RhoA-regulated genes. However, a detailed biochemical analysis of the MRTF-YAP interaction, the molecular mechanism of MRFA-YAP activity regulation and its functional impact in vivo are missing.

In their present manuscript Kim et al. analyzed the crosstalk between YAP and MRTF in vitro and in vivo. They defined the binding sites in both transcription factors and show that MRTF recruits NcoA3 to the YAP transcriptional complex to enhance its activity. Furthermore, the authors show the importance of the YAP-MRTF complex for LPA-induced cancer cell invasion in vitro and for the metastasis of 4T1 breast cancer cells in vivo. Finally, their study provides an explanation for the LATS-independent YAP activity regulation upon changes in the actin cytoskeleton.

The manuscript is well written and structured and provides a competitive study that strengthens our understanding of the functional mechanism of the YAP-MRTF transcriptional complex and provides an explanation for LATS-independent YAP activity regulation in response to changes in the actin cytoskeleton. This manuscript contains several important findings that are of interest for the general readership of the Embo Journal. Still, some issues should be addressed prior to publication.
(1) Is YAP activity regulation through MRTF-B more potent compared to MRTF-A? The authors observe differences in the ability of MRTF-A and -B to activate the TEAD luciferase reporter (Fig 1) and to interact with HA-YAP (Fig 3) but do not comment on these differences.

(2) Figure 1H: It is surprising to see a complete block of CTGF expression on a transcriptional level but only partial reduction by western blot (Figure 1G, F). It is not apparent from the figure legend and figure which MRTF has been depleted in this experiment.

(3) Figure 3C: While the CTGF promoter pulls down all three proteins, MRTF-B and TEAD4 but not YAP also seem to interact with the TAGLN and ACTB promoters.

(4) Figure 3F: The authors describe a series of coIP assays with several YAP mutants that have been performed to locate the interaction motive to the WW domain. However, shown is only the WW deletion and a 94A mutation, which is not explained in the figure legend or text. What is the purpose of the 94A mutation? Furthermore, also the other YAP mutations should be shown.

(5) The use of MRTF mutants that are inhibited for SRF or YAP binding is essential but the mechanism of crosstalk between MRTF-SRF and MRTF-YAP binding remains unclear. A detailed analysis would be out of the scope of this manuscript but the cell-type specific differences and the differences between the effects of the respective mutants in the reporter assays versus endogenous promoters are confusing. A better arrangement of the figures and the accompanying text would help to guide the reader through figure 4.

(6) Figure 4B: Although the text says that the authors want to measure YAP target gene expression this experiment only shows the expression of HA (YAP), MRTF-A, -B and TAZ but no target gene expression. In addition, the HA blot should be exchanged with a lower exposure time, also the MRTF-A blot as the MRTF-A knockdown or expression cannot be appreciated in the current blot.

(7) Figure 4B, C: Accompanying figure 4 B;C the authors write that they "did not observe any differences in YAP or TAZ phosphorylation or abundance...". This should be re-phrased as they did not look at TAZ phosphorylation. Furthermore, YAP phosphorylation was only addressed in the serum-stimulated case and not in both conditions. The absence of differences in YAP phosphorylation is difficult to evaluate from the blot provided in Figure 4C. YAP phosphorylation in the YA/ΔPY condition cannot be seen. It would be better to determine the band intensities of pYAP and total YAP from different experiments and show the ration of pYAP/YAP in a bar graph.

(8) Figure 5: The authors show that overexpressed MRTF-B increased TEAD2/YAP-NcoA3 binding but they did not analyze if the TEAD2/YAP-NcoA3 interaction is dependent on MRTF. Do TEAD2/YAP interact with NcoA3 in the absence of MRTF (e.g. in their knockout cells)?

(9) Figure 6A, B: LAP stimulation seems to increase the YAP-MART interaction and their nuclear co-localization. This increase is very difficult to see in the provided blots and immunostainings. The authors should quantify the enhanced interaction (by quantifying the band intensities) and co-localization (Pearson's correlation coefficient).

(10) Figure 6C, D: The experiment shows that LAP-induced invasion of T41 breast cancer cells depends on MRTF and that the MRTF mutation that cannot bind YAP does not rescue this defect. An additional control would be to also deplete YAP to verify that the ΔPY mutation indeed signals through YAP.

(11) Provide a statistical evaluation of the relative invasion. The Y305A construct only partially rescues the invasion defect. Is the difference significant?

(12) Figure 7C: Do the authors have an explanation why YAP 5SA interaction with MRTF-B ΔN is significantly weaker compared to the wt MRTF although YAP 5SA and MRTF-B ΔN are both retained in the nucleus?

Minor points:
(1) In many cases a proper description of the figure labels is missing in the figure legends. E.g. Figure 1G, 4C: include the explanation for the asterisk in the figure legend. Figure 1H,I: shMRTF - it is unclear if MRTF-A, -B or a combination of both has been depleted. Figure 2B: F-MRTFB is not explained (F = Flag-tagged?) and so on...

(2) Figure 1F: The Flag blot is overexposed but still Flag-YAP S5A seems higher expressed in lane 2 which could explain the increased CTGF expression in this sample.

(3) Figure 1G: When compared to Figure 1F the TAZ blot looks very different. Why the different exposure times?

(4) Figure EV3A: Are the effect of MRTF-B ΔPY compared to wt significant?

(5) In the text the figure EV3C is wrongly described as EV2C.

Referee #3, additional comment:

I also agree with the other reviewers that Latrunculin B treatment, which results in actin depolymerization, is not sufficient to conclude that MRTF-YAP mediates the mechanotransduction signal. To strengthen this conclusion, additional mechanical stress conditions should be tested such as soft/stiff or sparse/dense conditions in cells with and without MRTF activity.

1st Revision - authors' response 30 October 2016

We sincerely appreciate the excellent comments from our reviewers, as they were most helpful in improving our manuscript. Please find our point-by-point response to each of their comments below in blue.

Reviewer #1:
In this report, the authors investigated the physical and functional interaction between MRTF and YAP. Both MRTF and YAP are transcription co-activators and similarly regulated by serum as well as actin cytoskeleton. The authors showed that MRTF and YAP physically associate with each other and positively contribute to YAP target gene expression. MRTF recruits NcoA3 to enhance YAP transcription activity. Reconstitution of the MRTF knockdown 4T1 cells with wild type MRTF rescued tumor metastasis while expression of a YAP binding defective MRTF mutant was not effective. The authors conclude that the MRTF and YAP interaction plays a role in YAP target gene expression and in response to mechanotransduction. It is also concluded that MRTF potentiates YAP transcription activity to stimulate tumor metastasis.

This study has nicely demonstrated a functional interaction between MRTF and YAP, particularly on the recruiting of NocA3 in YAP dependent transcription activation. In addition, it also provides possible explanation about the LATS independent YAP target gene regulation in response to reorganization of actin. The implication of MRTF and YAP interaction in 4T1 tumor metastasis is potentially interesting. However, it should be noted that recent studies have shown that MRTF can functionally and physically interact with YAP and TAZ (Yu et al 2015; Speight et al 2016).
Specific comments

1) In order to conclude that "MRTF potentiates YAP-TEAD transcriptional activity for cancer metastasis", the authors need to show that YAP is critical for lung metastasis of 4T1 cells. The PPXY motif in MRTF may bind to other WW domain proteins important for metastasis. It needs to show that YAP WW domain mutant cannot support metastasis of 4T1, and regaining of MRTF interaction via a second site compensatory mutation restores the tumor metastasis. Without such data, the title is overstated and should be modified.

We appreciate the reviewer's thoughtful comment. Previous studies have shown that deletion of YAP WW domain does not impair metastasis (Lamar J. et. al., PNAS, 2012) using 67NR breast cancer model, which is syngeneic with 4T1 model. While WW domain deletion abrogates YAP binding to MRTF, thereby weakening YAP activity, this deletion will also abrogate YAP binding to negative regulators, such as angiomotin (Zhao B. et. al., Genes Dev. 2011), thereby activating YAP. We thus believe that YAP WW domain deletion will cause multiple disruptions in YAP regulations, both positive and negative, so that the net change in YAP activity may not be evident.
As an alternative, to confirm the role of YAP/TAZ in MRTF-mediated cancer metastasis, we infected MRTFA and MRTFB-depleted 4T1 cells with control or YAP/TAZ-specific shRNAs. Then, we added back either wild-type MRTFB or ΔPY MRTFB and performed *in vitro* invasion assays and *in vivo* metastasis assays. While wild-type MRTFB promotes more invasion and metastasis than ΔPY MRTFB in a YAP/TAZ-proficient background, neither form of MRTFB promotes invasion and metastasis in a YAP/TAZ-deficient background (Figure 6E, 6J and EV6). This suggests MRTFB-mediated cancer metastasis requires YAP/TAZ and is evidence that

YAP/TAZ-MRTFB binding itself promotes metastasis. We added these results to the revised manuscript (see page 12 lines 14-19, and page 13 lines7-13). We hope this will be sufficient.

2) The second sentence in the abstract "It is unclear, however, whether there is any crosstalk between these two proteins (MRTF and YAP)" is inaccurate. The Yu 2015 and Speight 2016 papers clearly examined the interaction between MRTF and YAP/TAZ. Actually, it has been shown that YAP and MRTF cooperate in gene expression and the TAZ WW domain is responsible for MRTF interaction.

We appreciate this correction. When we wrote this, the papers in question had not yet been published. We have now modified the abstract to indicate that while crosstalk is now known to occur, the exact mechanism remains unclear (see page 2, lines 2-5).

3) Fig.3.D. The data is a bit strange. YAP pulldown followed by MRTF IP retains little TEAD whereas YAP pulldown followed by TEAD IP retains significant MRTF. Do the authors have any explanation?

The two sequential IP results in the original manuscript were the results of independent experiments in which we recovered different amounts of prey protein after the IP. We have repeated the experiment and replaced the blot from the original Figure 3D with a new one that shows more consistent results.

4) Fig.5. The effect of NcoA3 knockdown on endogenous YAP target gene, such as CTGF and Cyr61, should be determined to demonstrate that NcoA3 affects YAP transcription activity. Does MRFT knockdown affect the interaction between YAP and NcoA3?

As suggested, we have generated MDA-MB-231 cells expressing either control or NcoA3 shRNAs and measured their expression of a few select TEAD-YAP target gene. As expected, we found NcoA3 depletion reduces TEAD-YAP target gene expression (Figure 5G). We also performed TEAD-NcoA3 and YAP-NcoA3 co-IPs with and without MRTFA/B depletion. Consistent with what we reported in the original version of this manuscript, we found MRTFA/B depletion attenuates NcoA3-YAP/TEAD binding (Figure 5J-K). These results suggest MRTF enhances TEAD/YAP-NcoA3 interactions to induce TEAD-YAP target gene expression. We have added these new data to the results section of the revised manuscript (see page 11, lines 15-16, and 21-22).

5) Fig.6A. The interaction between YAP and MTRF is not convincing. Can the authors repeat the experiments with each treatment having independent duplicated samples?

As suggested, we repeated this co-IP experiment and replaced the blot from Figure 6A with a clearer one. The MRTF-YAP interaction was very weak at endogenous level, we changed the co-IP experiment with exogenous MRTF and YAP to clearly show the increase in binding.

6) Fig.7C. Lat. B treatment causes actin depolymerization. Based on this data, it is not sufficient to conclude that MRTF-YAP mediates the mechanotransduction signal. In order to make such a conclusion, more mechanical stress conditions should be included in the experiments.

We agree with this insightful point from Reviewer 1. We added soft/stiff ECM conditions to our experiments for further evidence that the expression of YAP 5SA in the MRTF-knockout background renders TEAD-YAP target gene expression resistant to mechanical stress. Surprisingly, both wild type cells and MRTF-null cells expressing YAP 5SA in soft/stiff ECM sparse/dense conditions show similar reduced TEAD-YAP target gene expression by less tensile environment (Figure EV7C-D). As alternatives, we tested the other actin-disrupting drugs blebbistatin and Y-27632. Consistent with the findings with Latrunculin B, we did not see the reduction in TEAD-YAP target gene expression when we treated MRTF-null cells expressing YAP 5SA with these drugs (Figure EV7A-B).

We found that both disruption of MRTF-YAP binding and YAP phosphorylation by LATS render TEAD-YAP target gene expression completely refractory to acute actin disruption by depolymerizing drugs. However, combined disruption is still insufficient to block the decrease in TEAD-YAP target gene expression associated with long-term actin disruption (especially in the

form of prolonged high-density culture conditions or soft matrix culture conditions). We believe another slow-acting mechanotransducer may be present that is reducing YAP activity in the case of long-term actin disruption. Nevertheless, our results clearly show MRTF-YAP binding is an important regulator of YAP activity upon acute cytoskeletal insult. In the revised manuscript, we describe these new results on page 14, lines 12-13, 18-24.

We apologize that we were unable to explore the effect of acute changes in cell density or matrix stiffness because these are technically difficult to achieve. Still, we hope the alternative experiments we were able to perform will be sufficient.

Reviewer #2:
Kim et al report a functional interaction between YAP and MRTF. They demonstrate direct binding between YAP and MRTF, identify the interaction motifs, and test the functional importance of this interaction on regulation of transcription by YAP using in vitro luciferase reporter assays. Using different versions of MRTF that interact with YAP but not with its canonical binding partner SRF and vice versa, they show that the interaction between MRTF and YAP is essential for the regulation of YAP activity.

This is a thorough study with many innovative and well-performed experiments. The conclusions follow from the data and the finding that YAP activity depends on MRTF is highly interesting for the fields of mechanobiology and YAP/Hippo signaling. I only have a couple of suggestions to further test the contribution of SRF:

1. The authors use a truncated ctgf-Luc reporter as a reporter that depends on Yap-TEAD but not MRTF-SRF. In addition to this, the authors should use the 8xGTIIC reporter that contains only TEAD binding sites. This experiment is required to exclude binding by an SRF-MRTF complex to reporter constructs.

We apologize for causing confusion, but the experiment we performed for Figure 1A did use a reporter with 8 tandem TEAD binding sites upstream of luciferase. We have modified the results section and figure legends to clarify our use of an SRF-independent, TEAD activity reporter (see page 5, line 19-20, page 31 line 18).

2. In addition, the authors should repeat these experiments in an SRF mutant cell line (generated by CRISPR). Given the prominence of the SRF-MRTF complex in our current model of MRTF function, these experiments will be able to rule out/in SRF as a mediator of the MRTF effects on TEAD reporters.

In response to this suggestion, we performed TEAD luciferase reporter assays and SRF luciferase reporter assays on 293T cells in the presence and absence of both MRTFB over-expression and SRF depletion (Figure 1I). While SRF depletion attenuates the SRF reporter in the presence of MRTFB over-expression, it does not affect the TEAD reporter. This confirms that MRTF-mediated activation of TEAD-YAP is independent of SRF.

3. The authors argue that MRTF mediates the mechanical regulation of YAP. However, ECM stiffness for example has a major effect on Yap localization. How is MRTF supposed to be the mediator of the mechanical regulation of Yap when it does not affect the same phenotypes? I suggest that the authors assay the localization of Yap under soft/stiff or sparse/dense conditions in cells with and without MRTF activity. This will determine whether the mechanical regulation of Yap requires MRTF.
We appreciate this thoughtful comment. We have shown that in Lats-knockout cells, Latrunculin A-mediated actin disruption reduces TEAD-YAP target gene expression (Figure 7D) even though YAP protein remains inside the nucleus (Figure 7A). This suggests nuclear retention of YAP is insufficient for full YAP activation. In other words, there are two layers to the mechanical regulation of TEAD-YAP. The first is LATS-mediated YAP phosphorylation, which alters YAP's subcellular localization. The second layer is independent of YAP localization. It comprises the binding of YAP with MRTF, which regulates YAP's transcriptional activity once it is in the nucleus. We have added new data confirming the independence of MRTF status and YAP localization upon actin disruption (Figure 7A).

Unexpectedly, MRTF-knockout cells expressing YAP 5SA show reductions in TEAD-YAP target gene expression even when cultured in a soft matrix or in high cell density (Figure EV7C-D). So, we tested the alternative actin-disrupting drugs blebbistatin and Y-27632. We found that these drugs do not reduce TEAD-YAP target gene expression in MRTF knockout cells expressing YAP 5SA (Figure EV7A-B).

Overall, we found combined disruption of MRTF-YAP binding and YAP phosphorylation by LATS render TEAD-YAP target gene expression refractory to acute actin disruption by depolymerizing drugs. However, the disruptions in both regulations was still not enough to block the decrease in TEAD-YAP target gene expression induced by the long-term actin disruption associated with prolonged high-density culture conditions or soft matrix culture conditions. We believe another slow-acting mechanotransducer may be present that is reducing YAP activity in the case of long-term actin disruption. Nevertheless, our results clearly show MRTF-YAP binding is an important regulator of YAP activity upon acute cytoskeletal insult. In the revised manuscript, we describe these new results on page 14, lines 12-13, 18-24.

We apologize that we were unable to fully characterize the effects of acute changes in cell density or matrix stiffness because these are technically difficult to achieve. We hope the alternative experiments we have performed will satisfy Reviewer 2.

Reviewer #3:
The ability of cells to integrate and respond to a plethora of extracellular and intracellular information relies on transcription factors, which translate this information into precise cellular responses. YAP/TAZ are transcriptional coactivators in the Hippo pathway that shuttle between the cytoplasm and the nucleus, where they interact with other transcription factors - most notably TEA domain family members (TEAD) - to bind cis-regulatory elements. Mechanical cues represent a central mode for YAP/TAZ activity control. YAP and TAZ are directly regulated by ECM stiffness, cell shape, and cytoskeletal tension. SRF transcriptional coactivators of the Myocardin-related transcription factor (MRTF) family also respond to changes in the actin cytoskeleton and RhoA activation and previous studies revealed a crosstalk between YAP and MRTF. This functional relationship includes the activation of TAZ transcription by SRF-MRTF and the interaction of MRTF-A and YAP to promote RhoA-regulated genes. However, a detailed biochemical analysis of the MRTF-YAP interaction, the molecular mechanism of MRFA-YAP activity regulation and its functional impact in vivo are missing.

In their present manuscript Kim et al. analyzed the crosstalk between YAP and MRTF in vitro and in vivo. They defined the binding sites in both transcription factors and show that MRTF recruits NcoA3 to the YAP transcriptional complex to enhance its activity. Furthermore, the authors show the importance of the YAP-MRTF complex for LPA-induced cancer cell invasion in vitro and for the metastasis of 4T1 breast cancer cells in vivo. Finally, their study provides an explanation for the LATS-independent YAP activity regulation upon changes in the actin cytoskeleton.

The manuscript is well written and structured and provides a competitive study that strengthens our understanding of the functional mechanism of the YAP-MRTF transcriptional complex and provides an explanation for LATS-independent YAP activity regulation in response to changes in the actin cytoskeleton. This manuscript contains several important findings that are of interest for the general readership of the Embo Journal. Still, some issues should be addressed prior to publication.
(1) Is YAP activity regulation through MRTF-B more potent compared to MRTF-A? The authors observe differences in the ability of MRTF-A and -B to activate the TEAD luciferase reporter (Fig 1) and to interact with HA-YAP (Fig 3) but do not comment on these differences.

This is a valuable comment. We found that MRTFA and MRTFB show different affinities for SRF and YAP. MRTFB shows a lower affinity for SRF but a higher affinity for YAP (Figure EV3B). This is consistent with a previous study that performed an EMSA to demonstrate more efficient formation of the SRF-MRTFA complex than the SRF-MRTFB complex (Wang D-Z. et. al. PNAS, 2002). We also found that while MRTFB activates an SRF luciferase reporter at levels two-fold higher than MRTFA does, it activates a TEAD-YAP luciferase reporter at levels nearly ten-fold higher than MRTFA does (Figure EV3C). Thus, we believe that while MRTFA and MRTFB are

somewhat functionally redundant, they do show differential activation of target gene expression. MRTFA preferentially activates SRF, and MRTFB preferentially activates YAP. We have added these results to the revised manuscript (see page 8, line 22 to page 9, line 6).

(2) Figure 1H: It is surprising to see a complete block of CTGF expression on a transcriptional level but only partial reduction by western blot (Figure 1G, F). It is not apparent from the figure legend and figure which MRTF has been depleted in this experiment.

We apologize for the confusion. In Figure 1F and 1G, we depleted either MRTFA or MRTFB, but not both. Only Figure 1H shows the result of a double knockdown of MRTFA and MRTFB. Thus, while MRTFA and MRTFB show some redundancy in activating TEAD-YAP target genes, the combined depletion of both genes produces an even more dramatic reduction in target gene expression. We have changed the labels in the figures (especially Figure 1H) to clarify things. We hope this satisfies Reviewer 3's concern.

(3) Figure 3C: While the CTGF promoter pulls down all three proteins, MRTF-B and TEAD4 but not YAP also seem to interact with the TAGLN and ACTB promoters.

We used the CTGF, TAGLN, and ACTB promoters as representative TEAD-binding, SRF-binding, and negative control DNA sequences, respectively. Although we agree there is some non-specific, background binding of MRTFB and TEAD4 to all of these DNA fragments, the CTGF promoter clearly showed much stronger and more specific binding of TEAD4, YAP, and MRTFB. We hope this satisfies Reviewer's concern.

(4) Figure 3F: The authors describe a series of coIP assays with several YAP mutants that have been performed to locate the interaction motive to the WW domain. However, shown is only the WW deletion and a 94A mutation, which is not explained in the figure legend or text. What is the purpose of the 94A mutation? Furthermore, also the other YAP mutations should be shown.

We used the S94A mutant to address the possibility that the TEAD-YAP interaction may influence the MRTF-YAP interaction. We did this because we also showed that all three proteins form a complex (Figure 3D). We have added this point to page 8, lines 8-10, 19-22 of the revised manuscript. Also, as suggested, we added the results of co-IP experiments with MRTFB and all the YAP truncation mutants (Figure EV3A). As reported in our original manuscript, YAP's WW domain is critical for MRTF binding.

(5) The use of MRTF mutants that are inhibited for SRF or YAP binding is essential but the mechanism of crosstalk between MRTF-SRF and MRTF-YAP binding remains unclear. A detailed analysis would be out of the scope of this manuscript but the cell-type specific differences and the differences between the effects of the respective mutants in the reporter assays versus endogenous promoters are confusing. A better arrangement of the figures and the accompanying text would help to guide the reader through figure 4.
As requested, we have rearranged the figure 4 so that the data is more understandable. We hope Reviewer 3 will agree that our new figure layout is easier to follow.

(6) Figure 4B: Although the text says that the authors want to measure YAP target gene expression this experiment only shows the expression of HA (YAP), MRTF-A, -B and TAZ but no target gene expression. In addition, the HA blot should be exchanged with a lower exposure time, also the MRTF-A blot as the MRTF-A knockdown or expression cannot be appreciated in the current blot.
We apologize for the confusion. In Figure 4B, we simply confirmed the expression levels of exogenous YAP, MRTFA, and MRTFB. We demonstrated target gene expression in Figure 4D. We agree that our original layout was confusing, so we merged Figures 4B and 4D (see the new Figure 4B). We think this makes it more clear that these are results from the same set of cells. We also improved the blots. We hope this satisfies Reviewer 3's concern.

(7) Figure 4B, C: Accompanying figure 4 B;C the authors write that they "did not observe any differences in YAP or TAZ phosphorylation or abundance...". This should be re-phrased as they did not look at TAZ phosphorylation. Furthermore, YAP phosphorylation was only addressed in the serum-stimulated case and not in both conditions. The absence of differences in YAP phosphorylation is difficult to evaluate from the blot provided in Figure 4C. YAP phosphorylation in

the YA/ΔPY condition cannot be seen. It would be better to determine the band intensities of pYAP and total YAP from different experiments and show the ration of pYAP/YAP in a bar graph.

We quantified the pYAP/YAP ratio to determine whether MRTF regulates YAP phosphorylation. We observed a decrease in pYAP/YAP ratio in cells expressing MRTFB Y305A/ΔPY mutant. While the causes of this difference may be of interest for future studies, as decrease in YAP phosphorylation is expected to activate YAP, this change does not explain the failure of this mutant MRTFB to rescue YAP activity. Thus, we did not pursue further into this. We noted this finding in figure EV4A and in result section (page 9 lines 18-20).

We were unable to detect phospho-TAZ because of the quality of the phospho-TAZ antibody. So, in the revised manuscript, (page 9 line 16-18) we make it clear that we examined both the phosphorylation and abundance of YAP but only the abundance of TAZ. We wanted to avoid using densitometry for blot quantification because it is not standard practice in the field, but the new blot in Figure 4C clearly shows that neither MRTF depletion nor MRTF over-expression change YAP phosphorylation levels. We hope this satisfies Reviewer 3.

(8) Figure 5: The authors show that overexpressed MRTF-B increased TEAD2/YAP-NcoA3 binding but they did not analyze if the TEAD2/YAP-NcoA3 interaction is dependent on MRTF. Do TEAD2/YAP interact with NcoA3 in the absence of MRTF (e.g. in their knockout cells)?

This is a critical point that was also raised by Reviewer 1. Consistent with the original manuscript, we found MRTFA/B depletion attenuates NcoA3-YAP/TEAD binding (Figure 5J-K). This suggests MRTF enhances TEAD/YAP-NcoA3 interactions to induce TEAD-YAP target gene expression.

(9) Figure 6A, B: LAP stimulation seems to increase the YAP-MART interaction and their nuclear co-localization. This increase is very difficult to see in the provided blots and immunostainings. The authors should quantify the enhanced interaction (by quantifying the band intensities) and co-localization (Pearson's correlation coefficient).

We repeated this co-IP experiment and replaced the blot in Figure 6A with a clearer one. The MRTF-YAP interaction was very weak at endogenous level, we changed the co-IP experiment with exogenous MRTF and YAP to clearly show the increase in binding. Although we did not see any change in co-localization frequency in Figure 6B, the subcellular location of the co-localization is clearly different. It appears in the cytoplasm of serum-starved cells and the nucleus of LPA-treated cells. We think this is functionally relevant because MRTF-YAP colocalization in the nucleus is expected to increase YAP transcriptional activity. We have modified the manuscript to emphasize this change in the location of the YAP/MRTF co-localization (see page 12, lines 5-6).

(10) Figure 6C, D: The experiment shows that LAP-induced invasion of T41 breast cancer cells depends on MRTF and that the MRTF mutation that cannot bind YAP does not rescue this defect. An additional control would be to also deplete YAP to verify that the ΔPY mutation indeed signals through YAP.

As suggested, we compared cellular invasion of 4T1 cells expressing either wild-type or ΔPY MRTFB in both a control and YAP/TAZ-depleted background. We found that wild-type and ΔPY MRTFB produce consistent differences in invasive potential in the control background but not in the YAP/TAZ-deficient background (Figure 6E). This suggests ΔPY MRTFB's defect in promoting invasion depends on YAP activity. We have added these new results to the revised manuscript (see page 12, lines 14-19).

(11) Provide a statistical evaluation of the relative invasion. The Y305A construct only partially rescues the invasion defect. Is the difference significant?

We have added a statistical analysis of the data in Figure 6D. The difference with which wild-type and ΔPY MRTFB promote cellular invasion (P=0.08) was much more significant than that between wild-type MRTFB and the Y305A MRTFB mutant (P=0.58).

(12) Figure 7C: Do the authors have an explanation why YAP 5SA interaction with MRTF-B ΔN is significantly weaker compared to the wt MRTF although YAP 5SA and MRTF-B ΔN are both retained in the nucleus?

We are also curious about this result. We consistently observed reduced binding of the MRTFB ΔN mutant to YAP, but the reason is unclear. Since TEAD transcriptional activity initiates a negative feedback loop that inhibits YAP(Park G. et. al., Oncotarget, 2016), it is possible that some unknown factors hyper-activated by MRTFB ΔN expression are inhibiting YAP-MRTF association and thus inhibiting YAP activity. We expect the mechanism underlying this change in protein binding will make an interesting subject for future studies. We briefly mention this possibility in the revised manuscript (see page 14, line 4-7).

Minor points:
(1) In many cases a proper description of the figure labels is missing in the figure legends. E.g. Figure 1G, 4C: include the explanation for the asterisk in the figure legend. Figure 1H,I: shMRTF - it is unclear if MRTF-A, -B or a combination of both has been depleted. Figure 2B: F-MRTFB is not explained (F = Flag-tagged?) and so on...

We have improved our figure labels and figure legends. We expect they will now satisfy Reviewer 3.

(2) Figure 1F: The Flag blot is overexposed but still Flag-YAP S5A seems higher expressed in lane 2 which could explain the increased CTGF expression in this sample.

We agree that the original blot seemed to show variation in the level of Flag-YAP expression. We have repeated the experiment and replaced the blot in Figure 1F.

(3) Figure 1G: When compared to Figure 1F the TAZ blot looks very different. Why the different exposure times?

According to the literature, the loss of MRTF proteins reduces TAZ expression. Since we were trying to show that MRTF proteins do not alter TAZ expression, we wanted the TAZ bands to be visible in all lanes. Because there was significant batch-to-batch variation in the YAP-induced repression of TAZ, we had to adjust the exposure times accordingly. Since the point of this figure is demonstrating that depletion of neither MRTFA nor MRTFB alters TAZ expression, we think the figures adequately support our claims.

(4) Figure EV3A: Are the effect of MRTF-B ΔPY compared to wt significant?

We repeated the experiment in EV3A to verify our claim and found MRTFB ΔPY induces the expression of SRF-MRTF targets at levels similar to MRTFB WT.

(5) In the text the figure EV3C is wrongly described as EV2C.

We have corrected this error.

Reviewer #3, additional comment:
I also agree with the other reviewers that Latrunculin B treatment, which results in actin depolymerization, is not sufficient to conclude that MRTF-YAP mediates the mechanotransduction signal. To strengthen this conclusion, additional mechanical stress conditions should be tested such as soft/stiff or sparse/dense conditions in cells with and without MRTF activity.

As suggested by all three reviewers, we performed an additional experiment to verify that YAP 5SA expression in the MRTF knockout background indeed renders TEAD-YAP target gene expression refractory to mechanical stress. We did this by examining TEAD-YAP target gene expression in soft and stiff ECM culture conditions or in sparse and dense conditions (figure EV7C-D). Unexpectedly, we found MRTF knockout cells expressing YAP 5SA show reduced TEAD-YAP target gene expression. As alternatives, we then tested other actin-disrupting drugs blebbistatin and Y-27632. In

this case, we did not see the same reduction in TEAD-YAP target gene expression in YAP 5SA expressing MRTF knockout cells (figure EV7A-B).

We found that both disruption of MRTF-YAP binding and YAP phosphorylation by LATS render TEAD-YAP target gene expression refractory to acute actin disruption by depolymerizing drugs. However, combined disruption of both was insufficient to block the reduction in TEAD-YAP target gene expression induced by the long-term actin disruption associated with prolonged high-density culture conditions or soft matrix culture conditions. We believe another slow-acting mechanotransducer may be present that is reducing YAP activity in the case of long-term actin disruption. Nevertheless, our results clearly show MRTF-YAP binding is an important regulator of YAP activity upon acute cytoskeletal insult. We have added these new results to the revised manuscript (see page 14, lines 12-13, 18-24).

We apologize that we were unable explore the effect of acute changes in cell density or matrix stiffness because these are technically difficult to achieve. Still, we hope the alternative experiments we were able to perform will be sufficient.

2nd Editorial Decision 21 November 2016

Thank you for submitting the revised version of your manuscript. It has now been seen by two of the original referees, whose comments are enclosed below. While referee #1 was not able to look into the manuscript again, I asked the other two referees to consider his/her concerns as well.

As you will see, both referee #2 and referee #3 find that the concerns have been sufficiently addressed and are broadly in favour of publication, pending satisfactory minor revision, and a few editorial issues concerning text and figures that I need you to address. Referee #3 states, that your claims on the mechanotransduction pathways involved are not sufficiently well supported by the current data. Thus, I ask you to revise your manuscript regarding the points raised by referee #3, and relativise your statements where appropriate.

Please submit a revised version of the manuscript using the link enclosed below, addressing the reviewers' comments.

REFEREE REPORTS

Referee #2:

My concerns have been addressed.
Referee #3:

With one exception Kim et al. addressed my suggestions and comments in a satisfactory manner. The claim by Kim et al. that MRTF-YAP mediates a mechanotransduction signal still lacks sufficient experimental evidence. The authors performed experiments with additional actin-disrupting drugs, which confirmed their previous finding with Latrunculin A showing that MRTF-YAP mediates a signal upon acute cytoskeletal disruption. However, direct evidence for an involvement of this pathway in mechanotransduction is lacking as experiments with soft and stiff ECM or sparse and dense culture conditions show a reduced YAP target gene expressing in the absence of MRTF. The authors propose that a slow-acting mechanotransducer may present this reducing YAP activity. The identification of this slow-acting mechanotransducer would be out of the scope of the present study. Therefore, I suggest re-writing the passages referring to the significance of MRTF-YAP binding in mechanotransduction (as for example in the abstract, in the results section or the synopsis) and describe the significance of acute cytoskeletal disruption for MRTF-YAP binding as a regulator of YAP activity.

Additional comment referee #2:

I agree with the suggestion of referee 3.

2nd Revision - authors' response 25 November 2016

We sincerely appreciate the excellent comments from our reviewers. Please find our point-by-point response to each of their comments below in blue.

Reviewer #3:

With one exception Kim et al. addressed my suggestions and comments in a satisfactory manner. The claim by Kim et al. that MRTF-YAP mediates a mechanotransduction signal still lacks sufficient experimental evidence. The authors performed experiments with additional actin-disrupting drugs, which confirmed their previous finding with Latrunculin A showing that MRTF-YAP mediates a signal upon acute cytoskeletal disruption. However, direct evidence for an involvement of this pathway in mechanotransduction is lacking as experiments with soft and stiff ECM or sparse and dense culture conditions show a reduced YAP target gene expressing in the absence of MRTF. The authors propose that a slow-acting mechanotransducer may present this reducing YAP activity. The identification of this slow-acting mechanotransducer would be out of the scope of the present study. Therefore, I suggest re-writing the passages referring to the significance of MRTF-YAP binding in mechanotransduction (as for example in the abstract, in the results section or the synopsis) and describe the significance of acute cytoskeletal disruption for MRTF-YAP binding as a regulator of YAP activity.

We thank the reviewer for clarifying our manuscript. As suggested by the reviewer, we clarified our stance by removing 'mechanotransduction' from most of the manuscript and emphasizing that MRTF-YAP binding is responsible for regulation of YAP activity by 'acute actin cytoskeletal disruption'. Our edits were made in page 2 line 10, page 13 line 20, page 14 line 12, page 15 line 4. We also made changes in synopsis. We hope this satisfies the reviewer.

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Corresponding Author Name: Dae-Sik Lim
Journal Submitted to: EMBO Journal
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

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1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - are tests one-sided or two-sided?
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  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	page 23
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3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	page 23
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4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	page 23
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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	page 19

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