JCB

MASTL Promotes Cell Contractility and Motility Through Kinase-independent Signalling

Maria Taskinen, Elisa Närvä, James Conway, Laura Hinojosa, Sergio Lilla, Anja Mai, Nicola De Franceschi, Laura Elo, Robert Grosse, Sara Zanivan, Jim Norman, and Johanna Ivaska

Corresponding Author(s): Johanna Ivaska, University of Turku

Review Timeline:

Submission Date:	2019-06-28
Editorial Decision:	2019-07-23
Revision Received:	2020-02-03
Editorial Decision:	2020-03-02
Revision Received:	2020-03-10

Monitoring Editor: Kenneth Yamada

Scientific Editor: Andrea Marat

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.)

DOI: https://doi.org/10.1083/jcb.201906204

July 23, 2019

Re: JCB manuscript #201906204

Prof. Johanna Ivaska
University of Turku
Turku Centre for Biotechnology
Tykistökatu 6
Turku 20520
Finland

Dear Johanna,

Thank you for submitting your manuscript entitled "MASTL Promotes Cell Contractility and Migration through Transcriptional Control of Actin Regulators" to JCB. The manuscript has been evaluated by expert reviewers, whose reports are appended below. As you can see from the appended reviews, even though the findings were considered novel and potentially interesting, there were major concerns raised by each of these three experts in research areas spanned by this manuscript. Although the specific points listed needing resolution did not always coincide (though 2 of the 3 reviewers raised key overlapping points), there was unfortunately also a notable lack of enthusiasm for the study expressed in the priority evaluations for the Editors, even if the specific concerns were to be resolved. Consequently, we regrettably do not have the level of reviewer support that we would need for further consideration and publication in JCB.

We do not want to completely close the door on this original study, however, if you have data that can resolve the key concerns. They include the extent of the mechanistic advances, including the roles of kinase activity and especially identifying more definitive molecular mechanisms of MASTL action in invasion with molecular analyses of MASTL, MRTF/SRF, and GEF-H1. Two of the reviewers also deemed 3D analyses necessary, and there were a number of specific points raised that would need resolving. The sum total of all of these and other requirements seem daunting to us unless you already have some of these types of information in hand.

We regret that the decision could not be positive and are concerned that the needed extensive additions of data and concepts may not be practical for you and your colleagues at this point. If you wish to expedite the publication of the current data, it would seem best to pursue publication at another journal. Nevertheless, if you are interested in resubmitting to the journal, we would be open to discussing an appeal and revision plan, on which we could get reviewer input, to ensure you do not embark on time- and resource-consuming experiments that may not be sufficient for reconsideration at the journal. Please note that, per journal policy, priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Ken

Kenneth Yamada, MD, PhD
Editor, Journal of Cell Biology

Melina

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The manuscript by Taskinen et al. describes a novel role for the microtubule-associated serine-threonine kinase (MASTL) as a regulator of contractility. MASTL has been typically associated with the regulation of the cell cycle so this would represent a novel function for this kinase. The authors found that depletion of MASTL increases spreading and reduces contractility and migration. Through transcriptome and proteomic studies, they also identify a series of contractility associated genes that are mostly downregulated when MASTL is silenced, including GEF-H1, NM-2B and TPM4 among others. This initial part of the manuscript is very thorough, and the authors methodically dissect the phenotype, initially looking at the role of adhesion, integrins and the effects of depleting MASTL on cell migration.
The second part of the paper, in which the authors try to understand how MASTL regulates contractility is not as comprehensive. The article shows that that MASTL regulates MRTF-A nuclear levels and thus SRF-mediated gene expression.

Major comments:
My main concern is about the mechanism of action of MASTL. The authors explore the possibility that the regulation of MRTF by MASTL depended on a change in the G/F actin ratio but found no evidence of this. There are no other efforts on trying to understand the mechanism. Surprisingly, the authors do not look at the role of the kinase domain in this process. Does a kinase dead MASTL rescues the KD phenotype? What about the nuclear localization of MRTF? Are there any substrate candidates that maybe mediating the translocation of MRTF? Interestingly MASTL is nuclear so there is also a possibility of MASTL interacting with MRTF, or modulating its nuclear import/export/retention.

The authors also mention some known mutations for MASTL, which have been associated to disease, as support for the potential physiological relevance of this new role for MASTL. It would be interesting to see whether those mutations affect role of MASTL in mitosis or its role in the regulation ofthe SRF-mediated gene expression.

Minor Details:
-It is not very clear why some experiments are performed in MDA-MB-231, whereas others are done in MCF-7 cells. There does not seem to be a justification for switching cell lines from one experiment to the next.
-It is interesting that vinculin levels are significantly reduced (but not paxillin), but focal adhesions

size doesnt seem to be affected. However, these difference may reflect differences in the maturation status of focal adhesions. Maybe that should be explored a little bit more by staining for vinculin. Also, even though the focal adhesion size is not affected in MASTL KD cells, the number of focal adhesions was not determined. The micropatterened surfaces would be a good setup to look at this in more detail.
-In Fig 7F, it looks like siMRTF did not change GEF-H1 levels. This is not discussed in the text.

Reviewer #2 (Comments to the Authors (Required)):

The manuscript by Taskien et al., explores the interphase role of the kinase MASTL, in breast cancer cell lines, MCF10a and MDA MB 231. Whilst previous studies (Yoon, 2018 Rogers 2018) have identified a role for MASTL in breast cancer invasion, proliferation, anchorage independent growth, cell-cell junctions, colony forming capacity, and in regulating the cytoskeleton both in breast cancer (Rogers 2018) and haemopoietic cells (Hurtado 2018), Taskien et al., presents findings supporting the identification of a potential mechanism describing how MASTL may mediate this.
They find that MASTL regulates the expression of cell contractility regulatory proteins via SRF-MRFT-A (Myocardin-related transcription factor-A), linking actin architecture to SRF-mediated gene transcription. They identify the expression of RhoGEF, GEF-H1, and the actin-binding proteins, Tpm4.2, and Myo10 is decreased in response to MASTL depletion. This drives the MASTL-depletion phenotype, whereby cells have decreased spreading and migration. Although their findings will be of interest to the fields of cell, cytoskeleton and cancer biology, I have reservations that this may not represent a sufficient advance over previous work published by Rogers et al., (Oncogene 2018) and would require additional data to support their mechanism.
The authors should address the following:
1) The effect of MASTL depletion on cell spreading was examined in Fig 1C using MDA MB 231 cells. The authors then chose the non-transformed line MCF10 to investigate the effects of MASTL depletion on cell-spreading (Fig 2A), under the rationale that MASTL plays a role in transformed and non-transformed cell lines. MCF10 exhibit endogenously low levels of MASTL in comparison to transformed cell lines such as MDA-MB-231 or MCF7 cells (see PMID: 26613407). The manuscript would benefit from additional rationale for the choice of cell line given the low levels of MASTL in MCF10a, and adding in images of MASTL high lines (MCF7, MDA MB 231), to figure 2A, B, C.
2) Given there are significant alterations in migration (Figure 6 in MDA MB 231), yet no effect of siMASTL on focal adhesion size or integrin activity (Fig 2, MCF10a), have the authors considered an effect on FA dynamics rather than size? Figure 6 would benefit from the addition of a panel of FA markers, strengthening their observations from Figure 2. Particularly as no other publications have investigated cell-matrix adhesion and the authors have a strong track record in this area. Are the FAs more stable in MASTL siRNA cells? Are there defects in adhesion assembly or disassembly rates? Would it be possible that whilst FA size may be unchanged, that phosphorylation of paxillin may be altered? I assume the rationale for a "spreading" or "attachment" assay was to examine FAa that are undergoing a dynamic turnover, (cf. a wound healing assay?). This could be strengthened by the addition of live-cell imaging, of cells co-expressing an actin reporter (F-tractin) and vinculin (or paxillin). Allowing for the observation of kinetics of these processes, rather than a static end point. Labeling cell spreading as "Adhesion kinetics" was slightly confusing given the authors investigate cell-matrix adhesion in a subsequent figure. This could be clarified by labeling it as "cell-spreading" or "cell attachment" kinetics.
3) MASTL and cell-cell junctions. MASTL is over-expressed in several epithelial cancers (Colon, breast), and has been linked to regulating B-cat/wnt (doi: 10.1186/s12943-018-0848-3. Uppada Mol Cancer 2018). Given vinculin plays roles in cell-cell adhesion as well as cell matrix adhesion, and the

authors report vinculin and actin-binding proteins are regulated by MASTL, they should investigate cell-cell contacts and the organisation/localisation of vinculin and b-cat at cell junctions? Particularly as Rogers et al., (Fig 4) reported overexpression of MASTL resulted in an alteration in cell-cell junctions, and a loss of contact inhibition growth, consistent with previous reports.
4) Although MASTL amplification is through to be responsible for its oncogenic roles, (rather than mutation), is the kinase activity of MASTL required for the MRTFA nuclear translocation? This would benefit clinical studies trying to target MASTL, in that targeting kinase activity may not be necessary (doi: 10.1038/s41598-018-23246-0). Previous reports have suggested that the kinase activity is important for in invasion - with expression of a kinase dead GWL resulting in invasion and migration similar to control cells (Vera et al., elife 2015).
5) Validation of the proteomic targets. The data demonstrating GEF-H1 over expression rescues MASTL depletion (Figure 4J), needs to be stronger. More than simply morphology/spreading. Can it rescue the migration or invasion defects? Additional images of GEF H1 rescue cells need to be supplied, as they look morphologically different to those presented in Figure 1G, where eGFP-MASTL rescues. Are the cells simply less spread? is this a partial rescue? Given all three of the targets observed in Fig 4 localise to specific structures (microtubules, and actin-myosin respectively), the manuscript would benefit from the addition of immunofluorescence of these targets, comparing control and siRNA MASTL MDA MB 231. This approach could also be utilised for the issues with MCF10A cells not exhibiting a clear immuno-blot band.
6) Given that MRTFA siRNA (a) phenocopies MASTL depletion (fig 7), including a decrease in vinculin, an increase in cell area, and (b) MASTL-depletion in MDA MB 231 results in inhibition of cell migration (Figure 6), and a decrease in invasion in 3D models (Rogers 2018). Can the authors demonstrate that the loss of an invasive phenotype, is via MRTFA-dependent nuclear translocation? i.e. In 3D invasion assays of MDA MB 231 cells, Rogers et al., 2018 reported depletion of MASTL resulted in a loss of pseudopodia extensions into the matrix (and smaller spheres; Fig 7 specifically), and a decrease into fibroblast organised collagen matrix plugs. Investigating the role of MRTFA in a 3D invasion model would strengthen this observation. One would expect that siRNA of MRTFA would result in a decrease of pseudopodia extensions, similar to the MASTL-depletion phenotype in MDA MB 231. Likewise, investigation of actin organisation in 3D in both MASTL and MRFTA-depleted MDA MB 231 cells, would strengthen this observation.
Minor comments
7) SILAC results- It was unclear if any previously identified targets of MASTL were identified in this approach?
8) Biological vs. technical repeats and n values. It was unclear how many independent repeats were performed of each experiment. A table of statistics would help clarify this, including, n values, what that n represents (i.e a cell?), and how many times it was repeated i.e we measured 45 cells per condition, per repeat. Data is representative of 3 independent experiments.
9) The role of MASTL in the regulation of the actin-cap and TAN-lines. Have the authors observed any changes in nuclear positioning in their model? acto-myosin fibres plan an important role in nuclear positioning, which can affect motility. The Wirtz lab (doi: 10.1242/jcs.144345) identified a dorsal contractile perinuclear actin cap in fibroblasts, that plays an important role in nuclear positioning for migration persistence. Schwartz et al., Sci Rep 2017 (DOI:10.1038/s41598-017-01324-z)also have
have clear images of the apical and basal acto-myosin arrangements, dysregulated by key contractome proteins.
If MASTL regulates the contractome, potentially, there could be effects on nuclear positioning via the key proteins identified to be regulated by MASTL/MRTFA nuclear transition.

10) additional reference should be included (Cetti et al., 2019)
https://doi.org/10.1016/j.canlet.2018.11.010

particularly if you look at their supplementary videos of siRNA MASTL in thyroid cancer lines, you can clearly observe the large flat morphology the authors refer to in figure 6.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript the authors choose to investigate whether the serine/threonine kinase MASTL affects cell adhesion and cell shape and then investigate the molecular basis for these effects. While these results are potentially interesting, some key links between MASTL and cell shape/adhesion are missing.

It is not clear why the authors chose to work on MASTL. Did it come up as a hit in an RNAi screen they carried out?
A key point that is missing in the manuscript is whether MASTL kinase activity is required for the phenotypes observed (e.g. by testing rescue with a kinase-dead mutant) and if so whether MASTL acts to phosphorylate MRTF-A/SRF and hence regulate their function. The proteomic studies could have included phospho-proteomic analysis, which would have helped identify potential MASTL substrates that contribute to its effects on cell adhesion and cell shape.

A major concern with the results is that they are all carried out with only two breast cell lines (MDAMB231 cancer and MCF10A non-cancer) in 2D. It is important to determine whether MASTL also affects cell shape/actin distribution/MRTF-A localization in a more physiologically relevant 3D environment.
Another concern is that most of the results are based on only one siRNA to MASTL. Although some experiments have been carried out with two different siRNAs, this should have been routine. They have two, but only test both in a few experiments whereas they should have been routinely both tested. In addition, only one siRNA for MRTF-A and SRF is used rather than at least two.

Other points:
1. Figure 1: What is the effect of GFP-MASTL overexpression alone on cell shape? Presumably the level of exogenous GFP-MASTL is higher in cells that express it than endogenous MASTL levels - what % of cells express GFP-MASTL 24 h after transfection? The GFP-MASTL localization to the nucleus should be commented on in the text describing this figure.
2. Figure 4K: It is essential to show the effect of GEF-H1 overexpression alone on cell shape and spreading, and whether knockdown of MASTL affects this response.
3. Statistical analysis: in experiments where 45 cells (15/experiment) have been analysed, it appears that the p values have been calculated considering all 45 cells as separate experiments, although this has not specifically been clarified in the text. Instead, they need to compare the reproducibility of the phenotype in each of the three separate experiments, as has been done in some places. In some figure panels, the number of cells analysed is missing from the figure legends (e.g. Figure 1C, how many cells, what are the error bars; Figure 5, how many cells were analysed on crossbow shapes)? All figure legends should be checked and this information added. Finally, instead of putting n.s. the absolute p values should be included on figures because this allows readers to define for themselves whether differences are 'significant'. The authors should remove the word 'significant' from the text and rather use 'reduced', increased'.
4. Some experiments are only carried out in MCF10A cells when the original data were generated in MDAMB231 cells. For example, Anillin expression needs to be analysed in MDAMB231 cells as well as MCF10A cells. What is the effect of SRF/MRTF-A depletion on MDA-MB231 cells?
5. Figure 3/4: The choice of genes to pursue further appears arbitrary. Other genes in the lists (Fig.

3B and D) are equally plausible as candidates to regulate cell shape. It is rare that proteome and transcriptome analyses show much overlap, because proteome analyses only identify the most abundant proteins and/or those with peptides that fly well in a mass spectrometer. Given that MASTL apparently affects gene expression it would be more logical to concentrate on the transcriptome analysis and pick those with the strongest difference in expression. Why pursue genes that are already known to have a role in regulating cell contractility rather than screen through the top transcriptome/proteome hits for something potentially novel?

6. Figure 7: Was vinculin identified in the transcriptome/proteome screens? Why does MRTF-A depletion not affect GEF-H1? What about other targets from the transcriptome/proteome screens that have been followed up e.g. tropomyosin 4.2 (TPM4) and nonmuscle myosin IIB (NM-2B)? What is the reproducibility of MRTF-A translocation to the nucleus in different experiments? Where is MRTF-A in MDAMB231 cells?

7. What is the effect of GEF-H1 silencing on MRTF-A translocation to the nucleus? Presumably it would affect G/F-actin ratio via RhoA, whereas the authors imply that the effect of MASTL on MRTF-A nuclear localization/retention is independent of G-actin levels.

Reviewer #1 (Comments to the Authors (Required)):

The manuscript by Taskinen et al. describes a novel role for the microtubule-associated serine-threonine kinase (MASTL) as a regulator of contractility. MASTL has been typically associated with the regulation of the cell cycle so this would represent a novel function for this kinase. The authors found that depletion of MASTL increases spreading and reduces contractility and migration. Through transcriptome and proteomic studies, they also identify a series of contractility associated genes that are mostly downregulated when MASTL is silenced, including GEF-H1, NM-2B and TPM4 among others. This initial part of the manuscript is very thorough, and the authors methodically dissect the phenotype, initially looking at the role of adhesion, integrins and the effects of depleting MASTL on cell migration.

We would like to thank the reviewer for these supportive comments and for appreciating the novelty of this work.

The second part of the paper, in which the authors try to understand how MASTL regulates contractility is not as comprehensive. The article shows that that MASTL regulates MRTF-A nuclear levels and thus SRF-mediated gene expression.

We agree that this aspect of the work would benefit from additional investigation. Prompted by the insightful suggestions of the reviewer, we have performed new experiments that have enabled us to better understand how MASTL regulates contractility and cell migration, as well as uncovering a regulatory role for MASTL. We now show that MASTL associates with MRTF-A and promotes nuclear retention and transcriptional activity (new Figures 9C-F).

Major comments:
My main concern is about the mechanism of action of MASTL. The authors explore the possibility that the regulation of MRTF by MASTL depended on a change in the G/F actin ratio but found no evidence of this. There are no other efforts on trying to understand the mechanism. Surprisingly, the authors do not look at the role of the kinase domain in this process. Does a kinase dead MSTL rescues the KD phenotype? What about the nuclear localisation of MRTF?

We agree that resolving the role of MASTL kinase activity for our phenotype was of crucial importance and we therefore followed the advice to study the impact of the MASTL kinase domain. We generated an siRNA-resistant kinase-dead MASTLG44S-EGFP construct (previously published kinase-dead mutation, ref) for rescue experiments. Interestingly, we find that MASTL regulation of cell morphology and SRF are kinase-independent. We find that kinase-dead MASTL rescues the MASTL silencing effects on cell spreading as efficiently as the wild-type kinase (new Figure 1G-H). In addition, both MASTL constructs accelerate serum-induced nuclear localisation of MRTF-A (new Figure 9B) in MCF10A cells and augment serum-induced SRF transcriptional activity in luciferase promoter activity assays (new Figure 8E). Therefore, we conclude that MASTL-mediated regulation of cell morphology and gene expression of cytoskeletal/contractility regulating SRF/MRTF-A target genes is kinase activity independent.

Are there any substrate candidates that maybe mediating the translocation of MRTF?

The most important known regulator of MRTF nuclear translocation is G-actin/F-actin ratio. There are multiple proteins that are known to influence the G-actin/F-actin ratio, such as (cofilin, gelsolin, profilin) and thereby the translocation of MRTF. Given that cofilin activity is controlled by a serine-phosphorylation, we tested if MASTL silencing alters cofilin phosphorylation or levels. This did not seem to be the case (Figure 1 for reviewer).
Another possible substrate candidate that we have tested is MRTF-A itself, as it becomes heavily phosphorylated upon serum stimulation. However, we did not observe any difference in the degree of serum-induced MRTF phosphorylation when comparing control and MASTL-silenced cells (Figure 2 for reviewer). Both of these observations would be in line with our observation that MASTL kinase activity is dispensable for MRTF-A regulation by MASTL.

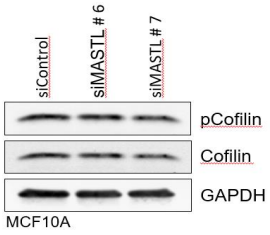


Figure 1 for reviewer #1. Phosphorylation of Cofilin. Western blot of total and phosphorylated Cofilin (Ser3) in Control siRNA or siMASTL transfected MCF10A cells.

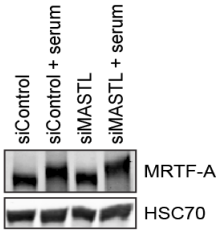


Figure 2 for reviewer #1. MRTF-A phosphorylation. Control siRNA or siMASTL transfected MCF10A cells cultured in 0.3 % FBS for 20 h followed by +/- stimulation with 20 % FBS for 30 min. MRTF-A phosphorylation was detected with a band-shift to a higher MW as described in (Panayiotou et al., 2016).

Interestingly MASTL is nuclear so there is also a possibility of MASTL interacting with MRTF, or modulating its nuclear import/export/retention.

We would like to thank the reviewer for this excellent suggestion that guided us to investigate the mechanism of how MASTL regulates MRTF-A. In line with this, we performed MASTL-EGFP pulldowns and blotted for MRTF-A, and found that both wild-type and kinase-dead MASTL pulldown/co-IP with MRTF-A (new Figure 9C). Furthermore, live-cell fluorescence loss in photobleaching (FLIP) experiments demonstrate that cytoplasmic relocation of GFP-MRTF-A out of the nucleus is accelerated in MASTL depleted cells, compared to control cells, indicating that MASTL-MRTF-A associations facilitate nuclear retention/accumulation of MRTF-A (new Figure 9D-F).

The authors also mention some known mutations for MASTL, which have been associated to disease, as support for the potential physiological relevance of this new role for MASTL. It would

be interesting to see whether those mutations affect role of MASTL in mitosis or its role in the regulation of the SRF-mediated gene expression.

We agree that testing the relevance of known MASTL mutations would add an exciting angle to our study. The MASTL E167D mutation, linked to human thrombocytopenia, was originally proposed to be a loss-of-function mutation (Johnson et al., 2009). However, a study in mice indicated that the corresponding E166D mutation does not prevent Mastl activity, and instead resulted in a gain-of-function; at least in the phosphorylation of putative PP2A-B55 substrates (Hurtado et al., 2018). Furthermore, the E166D mutation did not appear to affect mitosis (Hurtado et al., 2018). To assess this mutation in our systems, we generated a MASTL construct with the E167D point mutation and our data would be in-line with the notion that the E167D is a loss-of function mutation. We find that it does not accelerate nuclear translocation of MRTF-A (new Figure 9B), and does not augment serum-induced SRF promoter activity (new Figure S7D).

Minor Details:
-It is not very clear why some experiments are performed in MDA-MB-231, whereas others are done in MCF-7 cells. There does not seem to be a justification for switching cell lines from one experiment to the next.

We apologise for the lack of clarity in the manuscript, where key findings (cell spreading/GEF-H1) have been repeated with both of the cell lines initially used (MDA-MB-231 and MCF10A). However, we chose to study MRTF-A/SRF in MCF10A cells, as previous publications have indicated that serum starvation is not enough to induce MRTF-A translocation to the cytoplasm, in MDA-MB-231 cells (Medjkane et al., 2009), even though the basal MRTF-A/SRF activity in MDA-MB-231 cells is RhoA- and actin polymerization-dependent, and sensitive to MRTF-A-silencing (Medjkane et al., 2009). Therefore, the MCF10A cells were a more feasible model to study MRTF-A nuclear localisation, as a readout for SRF activity.
However, we are grateful to the reviewer for suggesting MCF7 cells as an additional model, as they have robustly reproduced our results and facilitated further investigation into the relationship between MASTL and MRTF-A. We have now used this cell line in multiple experiments to show that MASTL regulates SRF-promoter activity (new Figure 8E), cell spreading (new Figure S2B, C), MRTF-A association (new Figure 9C) and MRTF-A nuclear exit (new Figure 9D). We have also included additional text in the manuscript to better explain the rationale behind the chosen cell line models.

-It is interesting that vinculin levels are significantly reduced (but not paxillin), but focal adhesions size doesnt seem to be affected. However, these difference may reflect differences in the maturation status of focal adhesions. Maybe that should be explored a little bit more by staining for vinculin.

This is a great suggestion. We have stained vinculin in MCF10A cells. As expected, in MASTL-silenced cells, the focal adhesions contain less vinculin (new Figure S7B). We have also tracked focal adhesion turnover in live cells and find that MASTL silencing has no significant effect on focal adhesion dynamics (new Figure 7D-G). In addition, we assessed paxillin phosphorylation on Y118 and did not find any change in the levels of this marker of focal adhesion maturation stage (new Figure 7H, I).

Also, even though the focal adhesion size is not affected in MASTL KD cells, the number of focal adhesions was not determined. The micropatterened surfaces would be a good setup to look at this in more detail.

We have now determined the number of paxillin-positive focal adhesions (new Figure 2D). We find that MASTL silenced cells have more focal adhesions per cell, but when the number is normalized to the increased cell area, MASTL silenced cells actually have lower focal adhesion density per cell area.

-In Fig 7F, it looks like siMRTF did not change GEF-H1 levels. This is not discussed in the text.

This is correct. To our knowledge, GEF-H1 has not been described as an SRF target gene. GEF-H1 is a known inducer of SRF (Itoh et al., 2014; Ly et al., 2013) and therefore it is an upstream regulator of SRF and should not be affected by MRTF silencing. We have now added some additional discussion in the manuscript, related to the old figure 7F (now Figure 10C, page 21), and included the additional reference (Ly et al., 2013) to highlight this important point in the manuscript.

Reviewer #2 (Comments to the Authors (Required)):

The manuscript by Taskien et al., explores the interphase role of the kinase MASTL, in breast cancer cell lines, MCF10a and MDA MB 231. Whilst previous studies (Yoon, 2018 Rogers 2018) have identified a role for MASTL in breast cancer invasion, proliferation, anchorage independent growth, cell-cell junctions, colony forming capacity, and in regulating the cytoskeleton both in breast cancer (Rogers 2018) and haemopoietic cells (Hurtado 2018), Taskien et al., presents findings supporting the identification of a potential mechanism describing how MASTL may mediate this. They find that MASTL regulates the expression of cell contractility regulatory proteins via SRF-MRFT-A (Myocardin-related transcription factor-A), linking actin architecture to SRF-mediated gene transcription. They identify the expression of RhoGEF, GEF-H1, and the actin-binding proteins, Tpm4.2, and Myo10 is decreased in response to MASTL depletion. This drives the MASTL-depletion phenotype, whereby cells have decreased spreading and migration. Although their findings will be of interest to the fields of cell, cytoskeleton and cancer biology, I have reservations that this may not represent a sufficient advance over previous work published by Rogers et al., (Oncogene 2018) and would require additional data to support their mechanism. The authors should address the following:

We would like to thank the reviewer for an excellent and insightful review. We appreciate the concerns regarding the level of advance our data provides over previous work, particularly where Rogers et al., (Rogers et al., 2018) focused on describing phosphorylation changes linked to altered MASTL levels. In contrast, and prompted by the reviewers' suggestions, our new data indicates that MASTL regulates cell morphology and the cytoskeleton (MRTF-A/SRF transcription) independently of its kinase activity. The Rogers et al. study provides careful phosphoproteomic profiling of putative MASTL targets and concludes that "MASTL overexpression increased aberrant mitotic divisions resulting in increased micronuclei formation. Mathematical modelling indicated that this delay was due to continued inhibition of PP2A-B55, which delayed timely mitotic exit.", suggesting that

MASTL kinase activity is linked to MASTL oncogenic functions. Therefore, we are confident that our data describing kinase-independent functions of MASTL in regulating cell shape, contractility and expression of cytoskeletal proteins presents a significant advance in our understanding of MASTL as a major regulatory hub of cell morphology and migration.

1) The effect of MASTL depletion on cell spreading was examined in Fig 1C using MDA MB 231 cells. The authors then chose the non-transformed line MCF10 to investigate the effects of MASTL depletion on cell-spreading (Fig 2A), under the rationale that MASTL plays a role in transformed and non-transformed cell lines. MCF10 exhibit endogenously low levels of MASTL in comparison to transformed cell lines such as MDA-MB-231 or MCF7 cells (see PMID: 26613407). The manuscript would benefit from additional rationale for the choice of cell line given the low levels of MASTL in MCF10a, and adding in images of MASTL high lines (MCF7, MDA MB 231), to figure 2A, B, C.

We thank the reviewer for pointing out the possible difference in the endogenous levels of MASTL in different cell lines and for the suggestion to include MCF7 as a third cell line in this study. As suggested, we have compared endogenous MASTL levels in these cell lines (MCF10A, MDA-MB-231, MCF7) by western blot (new Figure S2A). Here we find that all three cell lines have readily detectable levels of MASTL, even though the levels in the MCF10A cells are the lowest (new Figure S2A). Further to this, we have now analysed the cell morphology of MCF7 cells upon MASTL silencing (new Figure S2B-C), highlighting the fact that we see a similar loss-of-function phenotype (increased cell spreading) in all of the cell lines upon MASTL silencing (MCF10A: new Figure 2A-B; MDA-MB-231: new Figures 1A-C, G, H, S1B, S1C, S1D, S1F, 5K; MCF7: new Figure S2B-C). Our data also show that despite MDA-MB-231 cells being “MASTL high”, overexpression of MASTL can further reduce cell spreading and that knockdown and rescue (with wild-type and kinase dead MASTL) can restore functionality in a kinase-independent manner (new Figures 1D,E,G-I). Moreover, silencing of MASTL in the “MASTL Low” MCF10A cells dramatically influences their phenotype as single cells (Figure 2A-B) and in confluent monolayers (new Figures 3, S7C). Taken together, this indicates that MASTL tunes cell morphology over a broad range of expression levels in different cell types in a kinase-independent manner.

2) Given there are significant alterations in migration (Figure 6 in MDA MB 231), yet no effect of siMASTL on focal adhesion size or integrin activity (Fig 2, MCF10a), have the authors considered an effect on FA dynamics rather than size? Figure 6 would benefit from the addition of a panel of FA markers, strengthening their observations from Figure 2. Particularly as no other publications have investigated cell-matrix adhesion and the authors have a strong track record in this area. Are the FAs more stable in MASTL siRNA cells? Are there defects in adhesion assembly or disassembly rates? Would it be possible that whilst FA size may be unchanged, that phosphorylation of paxillin may be altered? I assume the rationale for a "spreading" or "attachment" assay was to examine FAa that are undergoing a dynamic turnover, (cf. a wound healing assay?). This could be strengthened by the addition of live-cell imaging, of cells co-expressing an actin reporter (F-tractin) and vinculin (or paxillin). Allowing for the observation of kinetics of these processes, rather than a static end point. Labeling cell spreading as "Adhesion kinetics" was slightly confusing given the authors investigate cell-matrix adhesion in a subsequent figure. This could be clarified by labeling it as "cell-spreading" or "cell attachment" kinetics.

We agree that investigating the possible difference in focal adhesion dynamics is interesting and we have carried out live cell imaging of focal adhesion dynamics in MDA-MB-231 cells stably expressing a moderate level of mEmerald-Paxillin (Sahgal et al., 2019). Our detailed analyses of focal adhesion dynamics indicated that MASTL silencing has no significant effect on focal adhesion lifetime, nor on assembly or disassembly rates (new Figure 7D-G). In line with this observation, MASTL silencing has no significant effect on Paxillin phosphorylation status (new Figure 7H-I).

We apologize for the confusion with the term: adhesion kinetics. We have replaced "adhesion kinetics" with "cell attachment".

3) MASTL and cell-cell junctions. MASTL is over-expressed in several epithelial cancers (Colon, breast), and has been linked to regulating B-cat/wnt (doi: 10.1186/s12943-018-0848-3. Uppada Mol Cancer 2018). Given vinculin plays roles in cell-cell adhesion as well as cell matrix adhesion, and the authors report vinculin and actin-binding proteins are regulated by MASTL they should investigate cell-cell contacts and the organisation/localisation of vinculin and b-cat at cell junctions? Particularly as Rogers et al., (Fig 4) reported overexpression of MASTL resulted in an alteration in cell-cell junctions, and a loss of contact inhibition growth, consistent with previous reports.

This is a very interesting suggestion. As MDA-MB-231 cells do not form clear cell-cell contacts, we had not considered investigation of the cell-cell junctions. However, MCF10A cells are known to form clear cell-cell junctions and we have now investigated the effect of MASTL silencing on the junction components beta-catenin, E-cadherin and vinculin. Here we find that in the context of an epithelial monolayer of MCF10A cells, MASTL silencing significantly increases cell spreading, while E-cadherin, beta-catenin and vinculin remain in the cell-cell contacts (new Figure 3 and S7C). In addition, analysis of the orientation of beta-catenin-positive cell-cell junctions revealed no significant difference in the overall alignment of these junctions relative to each other (new Figure 3D,E and S3), despite obvious caps in the monolayer after MASTL silencing (new Figure 3B). In line with the fact that MASTL silencing reduces vinculin expression, vinculin intensity was reduced in the MASTL silenced cells, but still visible in the junctions (new Figure S7B,C). In addition, E-cadherin levels were analysed from MCF10A cells by western blotting. [redacted]

4) Although MASTL amplification is through to be responsible for its oncogenic roles, (rather than mutation), is the kinase activity of MASTL required for the MRTFA nuclear translocation? This would benefit clinical studies trying to target MASTL, in that targeting kinase activity may not be necessary (doi: 10.1038/s41598-018-23246-0). Previous reports have suggested that the kinase activity is not important for in invasion - with expression of a kinase dead GWL resulting in invasion and migration similar to control cells (Vera et al., elife 2015).

This is an excellent point. We agree that resolving the role of MASTL kinase activity for our phenotype is of crucial importance and we are grateful for this suggestion. To study this, we generated a siRNA-resistant kinase-dead MASTLG44S-EGFP construct (corresponding to previously published and validated kinase-dead mutation). Interestingly, we find that MASTL regulation of cell morphology and SRF are kinase independent. We find that kinase-dead MASTL rescues the MASTL silencing effects on cell spreading (new Figure 1G,H), as efficiently as the wild-type kinase. In addition, both MASTL constructs accelerate serum-induced nuclear localisation of MRTF-A (new Figure 9B) in MCF10A cells and augment serum-induced SRF transcriptional activity in luciferase promoter activity assays (new Figure 8E). Therefore, we conclude that MASTL-mediated regulation of cell morphology and gene expression of cytoskeletal/contractility regulating SRF/MRTF target genes in kinase activity independent.

In the Vera et al. study, the authors show that wild-type, but not kinase dead MASTL, induces MDA-MB-231 cell migration and invasion – the kinase dead MASTL overexpression has no effect and the cells behave as the control transfected cells. We have acknowledged this difference in their published data and our findings here in the discussion on page 24 by stating "morphology and motility. Previous work has indicated that kinase activity is necessary for MASTL overexpression induced migration of MDA-MB-231 cells (Vera et al., 2015), whereas we find that MASTL regulation of cell spreading and MRTF-A-SRF signaling are independent of kinase function. Currently, the reason for these somewhat discrepant findings is unclear."

5) Validation of the proteomic targets. The data demonstrating GEF-H1 over expression rescues MASTL depletion (Figure 4J), needs to be stronger. More than simply morphology/spreading. Can it rescue the migration or invasion defects? Additional images of GEF H1 rescue cells need to be supplied, as they look morphologically different to those presented in Figure 1G, where eGFP-MASTL rescues. Are the cells simply less spread? is this a partial rescue? Given all three of the targets observed in Fig 4 localise to specific structures (microtubules, and actin-myosin respectively), the manuscript would benefit from the addition of immunofluorescence of these targets, comparing

control and siRNA MASTL MDA MB 231. This approach could also be utilised for the issues with MCF10A cells not exhibiting a clear immuno-blot band.

The reviewer is correct that GEF-H1 only partially rescues the MASTL phenotype, presumably by working as a Rho GEF to increase cell contractility. This results in reduced cell spreading (new Figure 5J,K), but is not sufficient to rescue the reduced cell migration resultant from knockdown of MASTL (new Figure S6). Furthermore, we have provided new images of siControl and siMASTL transfected cells expressing either EGFP or EGFP-GEFH1 (new Figure S5C), where it is visible that the cells expressing GEFH1 are less spread, but their morphology is not identical to the control silenced cells.

To validate the proteomic targets, we have performed immunofluorescence for GEF-H1, Tpm4.2, NM-2B and vinculin. Tpm4.2 and vinculin levels are clearly decreased after MASTL silencing in MCF10A cells (new Figure S5D and S7C). In addition, GEF-H1 showed a clear reduction of signal intensity after MASTL silencing in MDA-MB-231 cells (new Figure S5B). Unfortunately, we were not able to validate NM-2B loss through immunofluorescence staining approaches.

6) Given that MRTFA siRNA (a) phenocopies MASTL depletion (fig 7), including a decrease in vinculin, an increase in cell area, and (b) MASTL-depletion in MDA MB 231 results in inhibition of cell migration (Figure 6), and a decrease in invasion in 3D models (Rogers 2018). Can the authors demonstrate that the loss of an invasive phenotype, is via MRTFA-dependent nuclear translocation? i.e. In 3D invasion assays of MDA MB 231 cells, Rogers et al., 2018 reported depletion of MASTL resulted in a loss of pseudopodia extensions into the matrix (and smaller spheres; Fig 7 specifically), and a decrease into fibroblast organised collagen matrix plugs. Investigating the role of MRTFA in a 3D invasion model would strengthen this observation. One would expect that siRNA of MRTFA would result in a decrease of pseudopodia extensions, similar to the MASTL-depletion phenotype in MDA MB 231. Likewise, investigation of actin organisation in 3D in both MASTL and MRFTA-depleted MDA MB 231 cells, would strengthen this observation.

This is definitely an area worth exploring in more detail, as depletion of MRTFs or SRF is known to reduce MDA-MB-231 cell invasion (Medjkane et al., 2009). Here we compared the effects of MRTF-A silencing to MASTL silencing on MDA-MB-231 cell invasion, and on the morphology/actin cytoskeleton of the cells in 3D. This work showed that MASTL and MRTF-A silencing have similar outcomes, where inhibition significantly reduced MDA-MB-231 cell invasion (new Figure 10D,E). Higher magnification microscopy of the cells invading in 3D also revealed that MASTL and MRTF-A silenced cells are significantly rounder than invading control cells, resembling the phenotype of MASTL silenced cells in 2D (new Figure 10F,G). Finally, MASTL silenced cells were found to have reduced nuclear MRTF-A, based on staining of endogenous MRTF-A from the invading cells in 3D (new Figure 10H,I).

Minor comments

7) SILAC results- It was unclear if any previously identified targets of MASTL were identified in this approach?

Phosphoproteomic analysis has been carried out previously by (Nagel et al., 2015) (MASTL silencing) and (Rogers et al., 2018) (MASTL overexpression). Of our most prominent hits (GEF-H1, TPM4, VCL, ANLN, MYH10), Nagel et al. identified a decrease in GEF-H1 (ARHGEF-2) phosphorylation and Rogers et al. detected phosphorylation of ARHGEF2 (S904), VCL (S721), ANLN (S485, S54) and MYH10 (S1965); however, the direction of change (up/down) was not indicated. We have also detected a change in the phosphorylation of GEF-H1 (Figure 2 for reviewer #2 below), but this most likely reflects the depletion of total GEF-H1 protein levels.

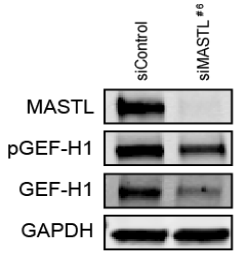


Figure 2 for reviewer #2. GEF-H1 phosphorylation. pGEF-H1 and total GEF-H1 levels in control and MASTL silenced MDA-MB-231 cells (n = 3).

8) Biological vs. technical repeats and n values. It was unclear how many independent repeats were performed of each experiment. A table of statistics would help clarify this, including, n values, what that n represents (i.e a cell?), and how many times it was repeated i.e we measured 45 cells per condition, per repeat. Data is representative of 3 independent experiments.

We agree that reporting statistics carefully is important. We have provided a table of statistics for clarification (Table S1).

9) The role of MASTL in the regulation of the actin-cap and TAN-lines. Have the authors observed any changes in nuclear positioning in their model? acto-myosin fibres plan an important role in nuclear positioning, which can affect motility. The Wirtz lab (doi: 10.1242/jcs.144345) identified a dorsal contractile perinuclear actin cap in fibroblasts, that plays an important role in nuclear positioning for migration persistence. Schwartz et al., Sci Rep 2017 (DOI:10.1038/s41598-017-01324-z)also have clear images of the apical and basal acto-myosin arrangements, dysregulated by key contractome proteins. If MASTL regulates the contractome, potentially, there could be effects on nuclear positioning via the key proteins identified to be regulated by MASTL/MRTFA nuclear transition.

We are grateful for this interesting suggestion. We have not observed any obvious differences in nuclear positioning. This might be an area to investigate in the future.

10) additional reference should be included (Cetti et al., 2019)
https://doi.org/10.1016/j.canlet.2018.11.010
particularly if you look at their supplementary videos of siRNA MASTL in thyroid cancer lines, you can clearly observe the large flat morphology the authors refer to in figure 6.

We thank the reviewer for calling our attention to this publication, which has now been highlighted in the revised manuscript text page 15.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript the authors choose to investigate whether the serine/threonine kinase MASTL affects cell adhesion and cell shape and then investigate the molecular basis for these effects. While these results are potentially interesting, some key links between MASTL and cell shape/adhesion are missing.

It is not clear why the authors chose to work on MASTL. Did it come up as a hit in an RNAi screen they carried out?

MASTL is an interesting kinase with potentially important functions that we wanted to investigate in breast cancer. This idea was further strengthened by the fact that MASTL scored as a putative integrin inhibitor in our unpublished integrin activity RNAi screen using PLA between active-β1 and integrin α-subunit antibodies as a read-out in prostate cancer cells. This prompted us to carefully explore whether MASTL would regulate integrin activity in breast cancer cells (Figure 2 and S2). However, as the data indicates, MASTL silencing increases cell surface levels of β1-integrins but does not directly regulate β1-integrin activity in MDA-MB-231 cells (Figure 2G,H, S2D-G).

A key point that is missing in the manuscript is whether MASTL kinase activity is required for the phenotypes observed (e.g. by testing rescue with a kinase-dead mutant) and if so whether MASTL acts to phosphorylate MRTF-A/SRF and hence regulate their function. The proteomic studies could have included phospho-proteomic analysis, which would have helped identify potential MASTL substrates that contribute to its effects on cell adhesion and cell shape.

We thank the reviewer for this excellent point. We agree that resolving the role of MASTL kinase activity for our phenotype was of crucial importance, and we are grateful for this suggestion. To study this, we generated an siRNA-resistant kinase-dead MASTLG44S-EGFP construct (corresponding to published and validated kinase-dead mutation). Interestingly, we find that MASTL regulation of cell morphology and SRF are kinase-independent. We find that kinase-dead MASTL rescues the MASTL silencing effects on cell spreading (new Figure 1G,H), as efficiently as the wild-type kinase. In addition, both MASTL constructs accelerate serum-induced nuclear localisation of MRTF-A (new Figure 9B) in MCF10A cells and augments serum-induced SRF transcriptional activity in luciferase promoter activity assays (new Figure 8E). Therefore, we conclude that MASTL-mediated regulation of cell morphology and gene expression of cytoskeletal/contractility regulating SRF/MRTF target genes is independent of its kinase activity.

Furthermore, we have tested whether MASTL is required for serum-induced MRTF-A phosphorylation, which involves multiple sites, most of which are S/T-P motifs (Panayiotou et al., 2016). However, we did not observe any difference in the degree of serum-induced MRTF phosphorylation when comparing Control and MASTL-silenced cells (Figure 1 for reviewer #3). This would be in line with the fact that MASTL effects are kinase independent.

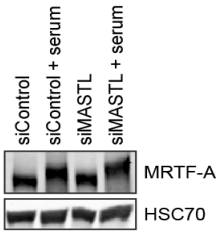


Figure 1 for reviewer #3. MRTF-A phosphorylation. Control siRNA or siMASTL transfected MCF10A cells cultured in 0.3 % FBS for 20 h followed by +/- stimulation with 20 % FBS for 30 min; n=3). MRTF-A phosphorylation was detected with a band-shift to a higher MW as described in (Panayiotou et al., 2016).

Published datasets describing MASTL-regulated effects on the cellular phosphoproteome (Nagel et al., 2015; Rogers et al., 2018) point to the existence of hundreds of putative direct/indirect MASTL targets. In addition, our data indicate that MASTL regulation of cell morphology and MRTF-A activity is not dependent on the MASTL kinase activity. Therefore, we consider that defining the MASTL substrate(s) involved in the regulation of MRTF is not relevant for this study.

A major concern with the results is that they are all carried out with only two breast cell lines (MDAMB231 cancer and MCF10A non-cancer) in 2D. It is important to determine whether MASTL also affects cell shape/actin distribution/MRTF-A localization in a more physiologically relevant 3D environment.

To address the reviewer's valid concern, we have performed key experiments with a third cell line; MCF7 cells. We have now used this breast cancer cell line in multiple experiments to show that MCF7 cells have comparable MASTL expression to MDA-MB-231 cells (new Figure S2A), which was also shown to regulate SRF-promoter activity (new Figure 8E), cell spreading (new Figure S2B,C), MRTF-A association (new Figure 9C) and regulate MRTF-A nuclear exit (new Figure 9D-F).
In addition, we have carefully explored the MASTL phenotype in a 3D environment. Depletion of MRTFs or SRF is known to reduce MDA-MB-231 cell invasion (Medjkane et al., 2009). Here we have compared the effect of MRTF-A silencing to MASTL silencing on MDA-MB-231 cell invasion and on the morphology/actin cytoskeleton of the cells in 3D and find that silencing MASTL or MRTF-A have inhibitory effects on MDA-MB-231 cell invasion (new Figure 10D,E). Higher magnification microscopy of the cells invading in 3D also revealed that MASTL and MRTF-A silenced cells are rounder than invading control cells (new Figure 10F,G). Finally, consistent with our 2D data (new Figure 9), MASTL silenced cells have reduced nuclear MRTF-A, based on staining of endogenous MRTFA from the invading cells in 3D (new Figure 10H,I).

Another concern is that most of the results are based on only one siRNA to MASTL. Although some experiments have been carried out with two different siRNAs, this should have been routine. They have two, but only test both in a few experiments whereas they should have been routinely both tested. In addition, only one siRNA for MRTF-A and SRF is used rather than at least two.

We agree that it is very important to control for off-target RNAi effects. Therefore, in most cases, we either had used two siRNAs or performed a rescue experiment; however, as much of the data with the second siRNA was included in the supplementary figures, it is possible that the information was

not clear to the reviewer. We apologise for this and outline below the experimental controls that were included:

- MASTL silencing and MDA-MB-231 cell spreading - two siRNAs (Figure 1A-B and S1B-D). In addition, we show a rescue experiment with MASTL-GFP wild-type in Figure 1G-H and have supplemented this with a new rescue experiment using the kinase-dead MASTL (new data in Figure 1G-H).
- MASTL silencing and integrin activity - two siRNAs (New Figure 2E-H and S2D-G)
- MASTL silencing effects on GEFH1, NM-2B and TPM4.2 - rescued with MASTL-GFP (old Figure 4I, now 5I).

In addition, the new experiments include data with two independent MASTL siRNAs: Cell spreading MCF7 cells (new Figure S2B-C), MCF10A cell monolayers and effects of junctional protein, vinculin and Tpm4.2 (new Figures 3A-E, S3, S5D and S7C), GEF-H1 in MDA-MB-231 cells (new Figure S5B) and GEF-H1 ability to revert cell spreading in MASTL silenced MDA-MB-231 cells (new Figures 5J,K and S5E).
We have also included a rescue experiment showing that re-expression of MASTL reverts reduced pMLC levels in MASTL silenced MDA-MB-231 cells (new Figure 6F).

SRF silencing (new Figure 7D) was included as a positive control for the SRF activity assay. This siRNA has been extensively validated by the Grosse group (Hinojosa et al., 2017), and this is now mentioned in the text page 17. In addition, the MRTF-A siRNA used in old 7F (now 10C) has been validated for specificity and efficacy previously (Hinojosa et al., 2017). However, in all the new experiments we have included two independent MRTF-A siRNAs (new Figure 10 D-I).

Other points:
1. Figure 1: What is the effect of GFP-MASTL overexpression alone on cell shape? Presumably the level of exogenous GFP-MASTL is higher in cells that express it than endogenous MASTL levels - what % of cells express GFP-MASTL 24 h after transfection? The GFP-MASTL localization to the nucleus should be commented on in the text describing this figure.

The data in Figure 1D-E demonstrates that MASTL overexpression induces a smaller cell morphology in MDA-MB-231 cells. In Figure 1F we show that the level of exogenously expressed GFP-MASTL (after siRNA knockdown and rescue) is similar to the endogenous MASTL levels in MDA-MB-231 cells. We have commented on the nuclear MASTL localisation in the text on page 19. This in fact is most likely relevant for the ability of MASTL to regulate MRTF-A. Our new data (new Figure 9C) indicates that MASTL and MRTF-A form a complex in cells and that MASTL supports nuclear retention of MRTF-A (new Figure 9D-F), and this has been highlighted in the discussion page 23..

2. Figure 4K: It is essential to show the effect of GEF-H1 overexpression alone on cell shape and spreading, and whether knockdown of MASTL affects this response.

Thank you for this suggestion. We have now imaged siControl and siMASTL transfected cells expressing either EGFP or EGFP-GEFH1 (new Figures 5J,K and S5E).

3. Statistical analysis: in experiments where 45 cells (15/experiment) have been analysed, it appears that the p values have been calculated considering all 45 cells as separate experiments, although this has not specifically been clarified in the text. Instead, they need to compare the reproducibility of the phenotype in each of the three separate experiments, as has been done in some places. In some figure panels, the number of cells analysed is missing from the figure legends (e.g. Figure 1C, how many cells, what are the error bars; Figure 5, how many cells were analysed on crossbow shapes)? All figure legends should be checked and this information added. Finally, instead of putting n.s. the absolute p values should be included on figures because this allows readers to define for themselves whether differences are 'significant'. The authors should remove the word 'significant' from the text and rather use 'reduced', increased'.

We agree that reporting statistics carefully is important. We provide a table of statistics for clarification (Table S1). We felt that the actual p-values for the n.s. labelled data were crowding the figures too much and thus we have provided the corresponding values in Table S1.

4. Some experiments are only carried out in MCF10A cells when the original data were generated in MDAMB231 cells. For example, Anillin expression needs to be analysed in MDAMB231 cells as well as MCF10A cells. What is the effect of SRF/MRTF-A depletion on MDA-MB231 cells?

This is a valid point and we have now analysed Anillin in MDA-MB-231 cells. Unexpectedly, MASTL silencing with two independent siRNAs did not significantly reduce Anillin levels. Currently we don't know the reason for this discrepancy with the proteomic data. However, since we are getting discordant data on MASTL regulation of Anillin between the two cell lines, we have decided to remove the anillin expression data from the manuscript. We are grateful to the reviewer for suggesting this important additional experiment.

Moreover, we have analysed the MCF10A-established effect of SRF/MRTF depletion in MDA-MB-231 cells in more detail (as mentioned also above). Depletion of MRTFs or SRF is known to reduce MDA-MB-231 cell invasion (Medjkane et al., 2009). So, we have compared the effect of MRTF-A silencing to MASTL silencing on MDA-MB-231 cell invasion, and on the morphology/actin cytoskeleton of the cells in 3D (new Figure 10D-I). Here we find that MASTL silencing and MRTF-A silencing have similar inhibitory effects on MDA-MB-231 cell invasion. Higher magnification microscopy of the cells invading in 3D reveals that MASTL and MRTF-A silenced cells are rounder than invading control cells. Finally, MASTL silenced cells have reduced nuclear MRTF-A based on staining of endogenous MRTFA from the invading cells in 3D (New Figure 10D-I).

5. Figure 3/4: The choice of genes to pursue further appears arbitrary. Other genes in the lists (Fig. 3B and D) are equally plausible as candidates to regulate cell shape. It is rare that proteome and transcriptome analyses show much overlap, because proteome analyses only identify the most abundant proteins and/or those with peptides that fly well in a mass spectrometer. Given that MASTL apparently affects gene expression it would be more logical to concentrate on the transcriptome analysis and pick those with the strongest difference in expression. Why pursue genes

that are already known to have a role in regulating cell contractility rather than screen through the top transcriptome/proteome hits for something potentially novel?

We appreciate that there are many different approaches to profiling data and in choosing the important pathway/s to investigate in detail. When we initiated these studies some years ago, the link between MASTL and the regulation of cell contractility was a fully unexplored area and thus the identification of several actin regulators as hits in our datasets was very interesting. As shown below (and in the supplementary tables of the manuscript) tropomyosin-4 (TPM4.2) and vinculin (VCL) were among the top hits.

The top 15 transcriptome hits are: SLC2A3 (-2.22), KYNU (-1.53), KYNU (-1.37), SH2B3 (-1.37), GTF2E2 (-1,33), CDK6 (-1.27), LOC151579 (-1.17), LOC203547 (-1.13), PACSIN2 (-1.08), DENR (-1.08), HIGD1A (-1.05), **TPM4 (-1.04)**, **VCL (-1.01)**, ESAM (-0.96) and PAPPA (+1.10).

The top 15 proteome hits are: SLC2A3 (-2.22), **TPM4 (-1.58)**, TMPO (-1.29), CEP97 (-1.28), DUSP11 (-1.26), KYNU (-1,25), **TPM4 (-1.24)**, CDK6 (-1.20), LBR (-1.19), BZW1 (-1.17), TTC38 (-1.13), UBASH3B (-1.11), SNTB2 (-1.07), DCP1A (-1.04) and ANLN (-1.04).

6. Figure 7: Was vinculin identified in the transcriptome/proteome screens?

Yes. Vinculin (*VCL*) was present in both transcriptome/proteome screens, as indicated in the new Figure 4B,C and in the Supplementary Tables S2 and S3.

Why does MRTF-A depletion not affect GEF-H1? What about other targets from the transcriptome/proteome screens that have been followed up e.g. tropomyosin 4.2 (TPM4) and nonmuscle myosin IIB (NM-2B)?

To our knowledge, GEF-H1 has not been described as an SRF target gene. GEF-H1 is a known inducer of SRF (Itoh et al., 2014; Ly et al., 2013) and therefore an upstream regulator, and should not be affected by MRTF silencing. In contrast, tropomyosin and myosin family members have been implicated as targets of the MRTF-SRF transcription pathway (Olson and Nordheim, 2010; Posern and Treisman, 2006).

What is the reproducibility of MRTF-A translocation to the nucleus in different experiments?

The experiment has been repeated three times and as shown by the error bars in the old Figure 7E (now 9A), these data are reproducible albeit there is some variation. We have expanded these studies further, by investigating the effect of wild-type, kinase-dead and E167D patient mutant MASTL on MRTF-A translocation and find consistently that wild-type and kinase dead MASTL increase the rate of MRTF-A nuclear translocation (new Figure 9B). Importantly, our new live-cell fluorescence loss in photobleaching (FLIP) experiments demonstrate that movement of GFP-MRTF-A out of the nucleus is accelerated in MASTL depleted cells, compared to control cells (new Figure 9D-F), indicating that MASTL supports nuclear retention of MRTF-A.

Where is MRTF-A in MDAMB231 cells?

It has been shown by the Treisman lab that serum starvation is not enough to induce MRTF translocation to the cytoplasm in MDA-MB-231 cells (Medjkane et al., 2009), although basal MRTF/SRF activity is RhoA- and actin polymerization-dependent, and sensitive to MRTF-silencing. Also in our hands, MRTF-A is nuclear in MDA-MB-231 cells, irrespective of serum stimulation (Figure 2 for reviewer #3). Therefore, MCF10A cells were used here to study MRTF-A nuclear localisation as a readout for SRF activity.

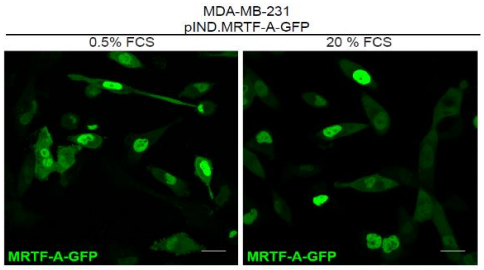


Figure 2 for reviewer #3. MRTF-A in MDA-MB-231s. Transfection of GFP-tagged MRTF-A into MDA-MB-231 cells shows nuclear localisation in serum starved (0.5 % FCS) or stimulated (20% FCS) conditions.

7. What is the effect of GEF-H1 silencing on MRTF-A translocation to the nucleus? Presumably it would affect G/F-actin ratio via RhoA, whereas the authors imply that the effect of MASTL on MRTF-A nuclear localization/retention is independent of G-actin levels.

Our new data indicate that MASTL regulation of MRTF-A involves the association of the two proteins (new Figure 9C) and that MASTL increases nuclear retention of MRTF-A in cells (new Figure 9D).

References

Hinojosa, L.S., Holst, M., Baarlink, C., and Grosse, R. (2017). MRTF transcription and Ezrin-dependent plasma membrane blebbing are required for entotic invasion. J. Cell Biol. *216*, 3087–3095.

Hurtado, B., Trakala, M., Ximénez-Embún, P., El Bakkali, A., Partida, D., Sanz-Castillo, B., Álvarez-Fernández, M., Maroto, M., Sánchez-Martínez, R., Martínez, L., et al. (2018). Thrombocytopenia-associated mutations in Ser/Thr kinase MASTL deregulate actin cytoskeletal dynamics in platelets. J. Clin. Invest. *128*, 5351–5367.

Itoh, K., Ossipova, O., and Sokol, S.Y. (2014). GEF-H1 functions in apical constriction and cell intercalations and is essential for vertebrate neural tube closure. J Cell Sci *127*, 2542–2553.

Johnson, H.J., Gandhi, M.J., Shafizadeh, E., Langer, N.B., Pierce, E.L., Paw, B.H., Gilligan, D.M., and Drachman, J.G. (2009). In vivo inactivation of MASTL kinase results in thrombocytopenia. Exp. Hematol. *37*, 901–908.

Ly, D.L., Waheed, F., Lodyga, M., Speight, P., Masszi, A., Nakano, H., Hersom, M., Pedersen, S.F., Szászi, K., and Kapus, A. (2013). Hyperosmotic stress regulates the distribution and stability of myocardin-related transcription factor, a key modulator of the cytoskeleton. Am. J. Physiol., Cell Physiol. *304*, C115-127.

Medjkane, S., Perez-Sanchez, C., Gaggioli, C., Sahai, E., and Treisman, R. (2009). Myocardin-related transcription factors and SRF are required for cytoskeletal dynamics and experimental metastasis. Nat. Cell Biol. *11*, 257–268.

Morita, T., Mayanagi, T., and Sobue, K. (2007). Dual roles of myocardin-related transcription factors in epithelial–mesenchymal transition via slug induction and actin remodeling. The Journal of Cell Biology *179*, 1027–1042.

Nagel, R., Walsum, M.S., Buijze, M., Berg, J. van den, Meulen, I.H. van der, Hodzic, J., Piersma, S.R., Pham, T.V., Jiménez, C.R., Beusechem, V.W. van, et al. (2015). Genome-wide siRNA Screen Identifies the Radiosensitizing Effect of Downregulation of MASTL and FOXM1 in NSCLC. Mol Cancer Ther *14*, 1434–1444.

Olson, E.N., and Nordheim, A. (2010). Linking actin dynamics and gene transcription to drive cellular motile functions. Nat. Rev. Mol. Cell Biol. *11*, 353–365.

Panayiotou, R., Miralles, F., Pawlowski, R., Diring, J., Flynn, H.R., Skehel, M., and Treisman, R. (2016). Phosphorylation acts positively and negatively to regulate MRTF-A subcellular localisation and activity. ELife *5*, e15460.

Posern, G., and Treisman, R. (2006). Actin' together: serum response factor, its cofactors and the link to signal transduction. Trends Cell Biol. *16*, 588–596.

Rogers, S., McCloy, R.A., Parker, B.L., Gallego-Ortega, D., Law, A.M.K., Chin, V.T., Conway, J.R.W., Fey, D., Millar, E.K.A., O'Toole, S., et al. (2018). MASTL overexpression promotes chromosome instability and metastasis in breast cancer. Oncogene *37*, 4518–4533.

Sahgal, P., Alanko, J., Icha, J., Paatero, I., Hamidi, H., Arjonen, A., Pietilä, M., Rokka, A., and Ivaska, J. (2019). GGA2 and RAB13 promote activity-dependent β1-integrin recycling. J Cell Sci *132*.

Seifert, A., and Posern, G. (2017). Tightly controlled MRTF-A activity regulates epithelial differentiation during formation of mammary acini. Breast Cancer Res *19*.

March 2, 2020

RE: JCB Manuscript #201906204R-A

Prof. Johanna Ivaska
University of Turku
Turku Centre for Biotechnology
Tykistökatu 6
Turku 20520
Finland

Dear Johanna,

Thank you for resubmitting your interesting manuscript entitled "MASTL Promotes Cell Contractility and Motility Through Kinase-independent Signalling", which was returned to the two expert reviewers. As you can see, they have relatively minor remaining concerns and suggestions. Consequently, we invite you to resubmit a final version of this manuscript after resolving as many of the remaining reviewers' points as practical, since addressing their final points should strengthen this excellent study. Please also ensure that your final manuscript meets our formatting guidelines for publication (see details below).

We hope you will agree that the in-depth reviewing by these conscientious reviewers will have resulted in a very strong final paper.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. * Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. *

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure

legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) * Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental display items (figures and tables). At the moment, you currently have 7 such items, please consider combining figures and be sure to correct the callouts in the text to reflect this change. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests

are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days, or advise us if you will need longer to address the final reviewer concerns.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

With kind regards,

Ken

Kenneth Yamada, MD, PhD
Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The manuscript by Taskinen et al. describes a novel role for the microtubule-associated serine-threonine kinase (MASTL) as a regulator of contractility. MASTL has been typically associated with the regulation of the cell cycle so this would represent a novel function for this kinase. The authors found that depletion of MASTL increases spreading and reduces contractility and migration. Through transcriptome and proteomic studies, they also identify a series of contractility associated genes that are mostly downregulated when MASTL is silenced, including GEF-H1, NM-2B and TPM4 among others. MASTL, which is nuclear loaclized, associates with MRTF-A and helps retain it in the nucleus. Importantly, these effects are independent of MASTL kinase activity.

In this revised version, the authors have diligently and thoroughly addressed all my concerns, and those of the other reviewers.
The new data extends the findings shown in the first version and adds mechanistic insights on the pathways regulated by MASTL.
I believe the new version of the manuscript represents a significant improvement.
I have no additional concerns.

I have only only a very minor comment:
In the resources table, the anti-b1 integrin Ab 12G10 is listed as anti-tubulin antibody (of the same clone number)

Reviewer #2 (Comments to the Authors (Required)):

Taskien et al., present additional data to address most of my points, and present logical rebuttals to the remaining points. As highlighted in their new manuscript, their revisions have discovered 1) MASTL regulates cellular architecture independent of its kinase function and 2) tunes cell morphology over a range of expression levels. These findings will impact our understanding of MASTL and its role in cellular architecture (which can correlate with metastatic capacity in solid tumours), and impact future drug design strategies.

Minor points to address before publication:
Addition of new cell line. Whilst the addition of MCF7 cells has strengthened the manuscript- could the authors address the discrepancies in band intensity between Fig S2A and Fig S5C? MCF10A MASTL levels look significantly lower in S2A vs 5SC, and the band pattern looks different. I understand it is difficult to adjust levels across blots, but the siControl MASTL band pattern in S5C looks very different to the MCF10a MASTL band in S2A.

MASTL and cell-cell junctions. (Re new Figure 3 and S7C). The monolayers look remarkably different-(great!)- a Z-stack of the monolayers, displayed as an orthogonal view may better display defects in cellular architecture. I would suggest the authors collect 0.2 um sections from basal to

apical, and measure the relative heights of the monolayer. This should be relatively easy and able to be collected on pre-existing slides/samples.

Tpm4.2 images
Could the authors clarify why the Tpm4.2 is cytoplasmic/punctate? Given it is incorporated into actin filaments (see figure 1e of https://doi.org/10.1038/s41598-019-42977-2). MCF10a (and MCF7) are stained for Tpm4.2 in this paper https://doi.org/10.1016/j.cub.2018.05.053 I would encourage the authors to revisit this, and co stain with phalloidin.

Pg 21 "elongated control silenced cells" should be "elongated control cells", as nothing is silenced.

10H grey scale images of MRTFA would be helpful, it is difficult to see the green MRTFA signal over the DAPI.

2nd Revision - Authors' Response to Reviewers: March 10, 2020

RESPONSE TO REVIEWER COMMENTS

Reviewer #1 (Comments to the Authors (Required)):

The manuscript by Taskinen et al. describes a novel role for the microtubule-associated serine-threonine kinase (MASTL) as a regulator of contractility. MASTL has been typically associated with the regulation of the cell cycle so this would represent a novel function for this kinase. The authors found that depletion of MASTL increases spreading and reduces contractility and migration. Through transcriptome and proteomic studies, they also identify a series of contractility associated genes that are mostly downregulated when MASTL is silenced, including GEF-H1, NM-2B and TPM4 among others. MASTL, which is nuclear loaclized, associates with MRTF-A and helps retain it in the nucleus. Importantly, these effects are independent of MASTL kinase activity.

In this revised version, the authors have diligently and thoroughly addressed all my concerns, and those of the other reviewers.
The new data extends the findings shown in the first version and adds mechanistic insights on the pathways regulated by MASTL.
I believe the new version of the manuscript represents a significant improvement.
I have no additional concerns.

I have only a very minor comment:
In the resources table, the anti-b1 integrin Ab 12G10 is listed as anti-tubulin antibody (of the same clone number)

We thank the reviewer for their positive comments and for pointing out the error regarding the antibody, which we have now corrected.

RESPONSE TO REVIEWER COMMENTS

Reviewer #2 (Comments to the Authors (Required)):

Taskien et al., present additional data to address most of my points, and present logical rebuttals to the remaining points. As highlighted in their new manuscript, their revisions have discovered 1) MASTL regulates cellular architecture independent of its kinase function and 2) tunes cell morphology over a range of expression levels. These findings will impact our understanding of MASTL and its role in cellular architecture (which can correlate with metastatic capacity in solid tumours), and impact future drug design strategies.

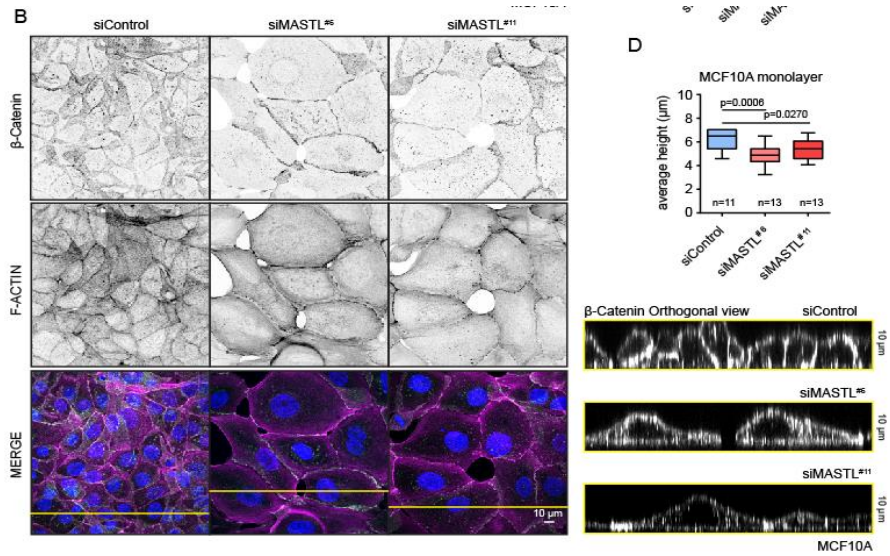
Minor points to address before publication:
Addition of new cell line. Whilst the addition of MCF7 cells has strengthened the manuscript- could the authors address the discrepancies in band intensity between Fig S2A and Fig S5C? MCF10A MASTL levels look significantly lower in S2A vs 5SC, and the band pattern looks different. I understand it is difficult to adjust levels across blots, but the siControl MASTL band pattern in S5C looks very different to the MCF10a MASTL band in S2A.

We are grateful to the reviewer for pointing this out. The different intensities are due to the fact that in Figure S2A the exposure was lowered to show MASTL levels in all three cell lines without overexposing the bands. Indeed, MASTL expression is higher in cancer cells (MDA-MB-231 and MCF7 cells) compared to normal cells (MCF10A cells). Figure S5C (now S4C) shows MASTL levels only in MCF10A cells and therefore the exposure could be set to optimally show the MASTL band in these cells. Regarding the band pattern, it seems that Fig S5C was more tightly cropped than S2A and thus the higher MW band detected in MCF10A with anti-MASTL antibody was not fully visible in Fig S5C. We have now replaced S5C with a less tightly cropped image. As can be seen from the two MASTL silenced samples in S5C, it seems that the higher MW band is unspecific as it is not affected by the siRNAs that strongly reduce the lower MW band.

MASTL and cell-cell junctions. (Re new Figure 3 and S7C). The monolayers look remarkably different- (great!)- a Z-stack of the monolayers, displayed as an orthogonal view may better display defects in cellular architecture. I would suggest the authors collect 0.2 um sections from basal to apical, and measure the relative heights of the monolayer. This should be relatively easy and able to be collected on pre-existing slides/samples.

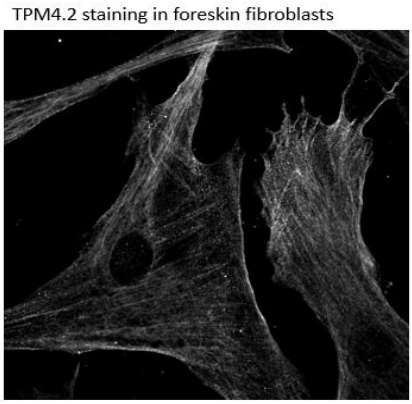
Thank you for the great suggestion. We have now added the orthogonal view of the monolayer to Fig. 3B. In addition, we have measured the relative height of the monolayer in 3D, which is significantly lower following MASTL silencing (figure below for your convenience).

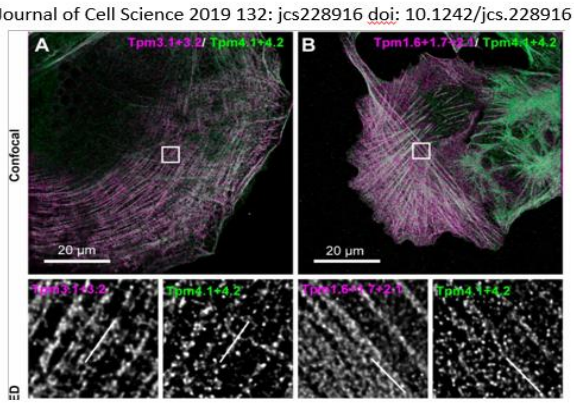
RESPONSE TO REVIEWER COMMENTS



Tpm4.2 images. Could the authors clarify why the Tpm4.2 is cytoplasmic/punctate? Given it is incorporated into actin filaments (see figure 1e of https://doi.org/10.1038/s41598-019-42977-2). MCF10a (and MCF7) are stained for Tpm4.2 in this paper https://doi.org/10.1016/j.cub.2018.05.053 I would encourage the authors to revisit this, and co stain with phalloidin.

The actin fibers on MCF10A cells https://doi.org/10.1016/j.cub.2018.05.053 pointed out by the reviewer are very prominent. In our experimental setup, the MCF10A cells do not seem to have such prominent actin fibers as shown in Fig. 2A, Fig. 3B and Fig. S4D. Therefore, we believe that our TPM4.2 staining would not resemble the strong fiber-staining pattern reported in these studies. We have validated the TPM4.2 antibody staining in foreskin fibroblasts (see left-hand panel below), which do have very prominent actin fibers and where TPM4.2 shows a clear fiber-associated staining pattern. In addition, as shown in the Journal of Cell Science 2019 132: jcs228916 doi: 10.1242/jcs.228916 TPM4.2 can appear punctate similar to our staining (see right-hand panel below).

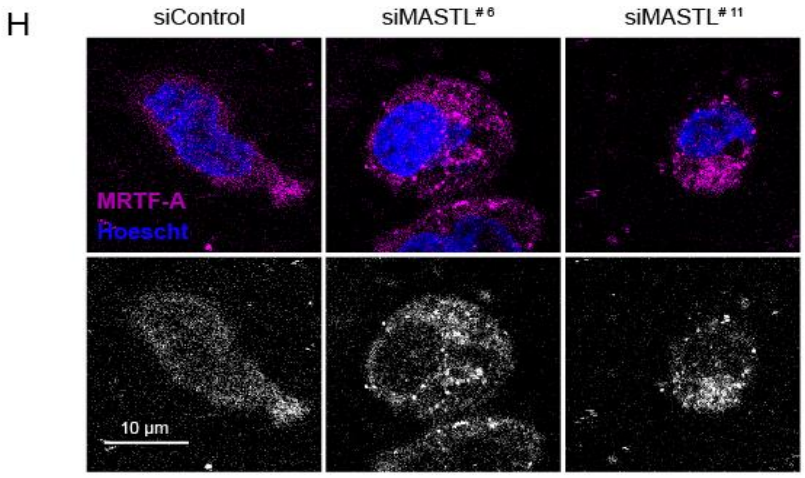




10H grey scale images of MRTFA would be helpful, it is difficult to see the green MRTFA signal over the DAPI.

RESPONSE TO REVIEWER COMMENTS

We have now added grey scale images of MRTF-A to Fig. 10H. In addition, we changed the blue/green combination to blue/magenta (figure below for your convenience).



Pg 21 "elongated control silenced cells" should be "elongated control cells", as nothing is silenced.

This has been corrected.