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Actin Remodeling Confers BRAF Inhibitor Resistance to Melanoma Cells through YAP/TAZ Activation

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(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

29 June 2015

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see below, the manuscript received a bit of a mixed response. Referee #2 is not persuaded that the advance provided is sufficient over the recent Lin et al. Nature Genetics paper. Referee #1 and 3 are more supportive, but they also raise other concerns. I do think that your manuscript makes an important contribution also in light of the recent Nature Genetics paper. However, there are issues with the study that would have to be resolved in order to consider publication here. The referees find that there are several inconsistencies in particular regarding the TESK1 dataset that needs to be sorted out. Their concerns are clearly outlined below.

Should you be able to extend the analysis along the lines indicated by the referees then we would be interested in considering a revised version. I should add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to resolve the raised issue at this stage.

I think in this case it would be productive to discuss upfront what experiments you can add within a reasonable timeframe. Could you send me a point-by-point response outlining what experiments you can do so we can discuss it further.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:

http://emboj.embopress.org/about#Transparent_Process

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

REFEREE REPORTS

Referee #1:

This is an interesting paper on a topic that has been already covered by a prior publication. That said, this is a significant advancement and the Nat medicine paper should not detract from the potential importance of this paper for the wide audience of EMBO J.

Gene expression. The authors are just focusing on 3 YAP/TAZ targets upregulated in resistant cells. The authors should be more convincing by deriving YAP/TAZ-dependent gene signature in melanoma cells (even better if resistant vs. non resistant cells; part of the work on YAP/TAZ dependency has been already done in fig 6) and compare it public datasets to infer the effects on survival in patients treated with B-raf inhibitors.

One problem of this and of the prior Nat Med paper is actually a difficult one. They focus on YAP/TAZ and imply that this is involved in resistance. However it is unclear to me if this is really specific. Given the pivotal role of YAP in cell proliferation, one wonders if YAP would surpass resistance of everything. I do realize that this is somewhat a "philosophical" concern. However, some better controls are still due. It is unclear for me if the effect of YAP, TAZ or combined YAP/TAZ depletions is really stronger (in fold induction) in resistant vs non-resistant cells. Parental cells have not been examined. While I suspect that YAP/TAZ inactivation may have just the same effects, there may be a "window" of specificity using the partial/single YAP or TAZ knockdowns.

Figure 7G and H. The regulation of protein levels by transfected LATS is problematic. YAP is hardly changed at the total protein level, questioning the specificity of these results. Moreover the Hippo pathway is regulated by LATS phosphorylation and not by reduced protein levels. Oppositely, new evidence in the field suggests the opposite: LATS kinases are typically induced by the active YAP as a negative feedback. Overall we have no clue on what regulate LATS levels in these cells. The YAP overexpression and subsequent speculation are thus irrelevant if not misleading. Finally, while it is clear that the cytomechanics and F-actin control YAP/TAZ, work from several groups - including the original papers from S.Piccolo group - failed to reveal a functional role of YAP phosphorylation by LATS downstream of F-actin and Cofilin (addressed in Fig 8). There are no definitive answers and the intersections with the cytoskeleton occur at many levels. All this clearly requires a set of dedicated studies. For the time being, I suggest simply to remove these results; they can eventually keep it in discussion as a speculative point.

Figure 2: the b-catenin staining is inconclusive. I see nuclear staining as well in one panel. Please substitute with a different membrane marker. Or with simple bright-fields for cell area/shape.

I can hardly take any serious conclusion from Fig. 3C.

siYAP/TAZ: figure 4 and all other panels. ALL the key experiments should be repeated with an independent pair of siRNAs and rescued with cDNA. The risk of offtarget effects is known.

The myc connection is quite intriguing and novel. Can Myc rescue siYAP/TAZ (re-establishing resistance)?

Fig. 8. One experiment to attempt is whether knockdown of Cofilin does the opposite of TESK in SKMEL and WM cells (i.e. induce resistance in non-resistant/parental cells). It may be asking for too much, but it works it would complete the panel and make a strong point on F-actin.

Minor notes

Page 5. 2nd line. the ref is Zhao et al 2012

Page 5 8XGTIIC-luciferase needs a reference. Why not call this a TEAD reporter?

Repeat the color legend in main figure Fig 8G
Why the effect of TESK knockdown is so weak in WM cells?

Discussion must be greatly simplified while remaining on the merit of the MS.
For example: The first paragraph is useless. Discussion on existing small molecules can be deleted. They were not tested in this context.

The discussion on Mori et al: as discussed I do not believe it is pertinent. They propose a transcription-independent mechanism in that paper. Is this what they imply for YAP/TAZ here? I doubt it, as they use CTGF as transcriptional read out. How c-Myc is regulated here remains undefined.

The discussion on Feng et al makes sense to the general reader only if it is clarified somewhere that Feng concludes that the cytoskeletal and Hippo/LATS cascades are distinct

Referee #2:

The authors analyze resistance to RAF inhibitors in mutant BRAF melanomas with a focus on the role of the actin cytoskeleton, YAP/TAZ and TESK1. While the study is well performed, concerns about the study being an incremental advance, not providing sufficient evidence for the role of TESK1, and lack of relevance to patient material lessens enthusiasm for the study.

1. Previous studies have implicated YAP/TAZ in resistance to RAF inhibitors (Lin et al 2015 Nature Genetics). While this study is noted, there is an incremental advance provided by the current study.
2. Other studies have implicated a role for extracellular matrix/integrin signaling in resistance to RAF inhibitors and changes in cell morphology/actin cytoskeleton (e.g. Hirati et al. 2015 Cancer Cell; Shao et al 2012 Cell Death Diff). Thus, the notion that actin cytoskeletal remodeling is linked to resistance is not new.
3. Multiple independent siRNAs need to be utilized per target (YAP/TAZ) to eliminate concerns about off-target effects.
4. What mediates the actin reorganization in the vemurafenib resistant cells is unclear. TESK1 is not upregulated in the resistant SKMEL28 cells. The majority of the data are shown with resistant WM3248. The frequency of alterations of TESK1 is not evident.
5. The use of the term adaptive resistance is unclear in the context of this manuscript.
6. TESK1 as a therapeutic target is unclear. The frequency of TESK1 alterations in human samples is unclear.

Referee #3:

The authors present a BRAF inhibitor resistance mechanism in which melanoma cells undergo profound changes in cellular morphology and increased actin cytoskeletal remodeling, resulting in greater YAP/TAZ translocation to the nucleus and transcriptional activity. The authors support this model by showing that perturbations that inhibit cytoskeletal remodeling result in reduced nuclear localization of YAP/TAZ and lower target gene expression. The functional importance of YAP/TAZ was supported by genetic experiments where knockdown of YAP/TAZ resulted in growth arrest and restored some PLX4032 sensitivity in resistant cell lines, and overexpression of a constitutively active YAP is sufficient to render parental cell lines resistant. Interestingly, increased YAP transcriptional activity results in down-regulation of MITF and SOX10 and up-regulation of EGFR, consistent with other previously identified resistance mechanisms.

A strength of the study is that it unifies prior findings to highlight the relationship between actin cytoskeletal remodeling, activation of transcriptional programs, and acquisition of drug resistance. The identification of YAP regulation of MITF is interesting, given the important role of MITF in melanoma biology. The authors perform an extensive array of experiments, some of which support their conclusions, some of which have notable (and unmentioned) discrepancies between cell lines, some of which are informative but do not clearly rule out an alternative possibility (upstream activation), and some of which seem no more than confirmatory (downstream gene expression analysis).

The authors were able to identify novel drug targets from actin dynamics regulators (TESK1) that can increase tumor cell death in combination with PLX4032. However, here again there are inconsistencies between the two cell lines used in that the TESK1 knockdown only works as expected in one line (Fig 8I), and for unaddressed reasons this result is not consistent with the biomarker (TESK1 expression levels) data for these cell lines (8C).

In summary, inconsistencies need to be addressed and understood, and additional cell lines need to be tested in order to support the authors conclusions.

Comments:

There are a number of discrepancies between the two cell lines, in some cases one cell line fits the model better and in other cases where this is reversed. While SKMEL28 is generally consistent with the author's claims when both cell lines are presented, it is unclear why only WM3248 is presented in others. There are difficulties reconciling these differences, and lack of acknowledgement and discussion of the discrepancies by the authors. Additional cell lines should be evaluated to strengthen the claims. The following 5 points relate to discrepancies between cell lines:

Knockdown of YAP in WM3248 resistant cells do not completely restore sensitivity in the WM3248 cell line (Fig 4A), with the highest concentration only decreasing relative survival to 50%. Yet, the manuscript text generalizes across the two cell lines "a remarkable restoration of sensitivity."

Across the two lines, the role of MYC is unclear. While resistance and active YAP5SA expression prevents PLX4032 mediated down-regulation of MYC, MYC levels also decrease with establishment of resistance in WM3248 cells (to levels even lower than in PLX4032-treated SKMEL28 cells) (Fig 1F).

There is no explanation as to why the results in Fig 4E are shown for only WM3248 cells. Fig 4E results are stated in the text with no interpretation or follow-up. What is the importance of these findings, especially in light of the low MYC levels in resistant WM3248 cells (Fig 1F)?

The microarray gene expression experiment in Fig 6 is lacking the use of a parental control line to demonstrate that the observed enrichment results are only present in resistant cells. It is unclear why the WM3248 line was chosen for these studies. Profiling both cell lines would strengthen the arguments. The focus on MYC in this section further raises the question on the observed substantial decrease in MYC protein levels in resistant WM3248 but not resistant SKMEL28 (1F).

The author's state "As expected, the reduction of cell viability after TESK1 knockdown was higher in resistant SKMEL28 and WM3248 cells than the reduction in parental cells (Fig 8I)" but in this discussion they do not address the fact that in the WM3248 parental cells, the reduction in viability by TESK1 knockdown was very large, which is in stark contrast to the effect in SKMEL28 parental cells. Furthermore, the results of Fig 8C have a reversal in regards to which cell line shows a difference between parental and resistant sublines, this time in terms of TESK1 expression levels, which is difficult to reconcile with the opposing trends seen in 8I. Why is the biomarker pattern not predictive of the knockdown response?

The authors state that "Survived resistant clones were maintained in the presence of 2 uM PLX4032." However, no details are given regarding the timing of drug removal and subsequent exposure when resistant cells were used in experiments that compared with and without PLX4032 conditions. These aspects are critical due to rebound effects and kinetics of drug removal and exposure, especially for pERK. Signs of such effects are seen in the increase growth of one line when low doses of PLX4032 are present (Fig 1A, 4A). The absence of such detail, and explanation of consideration given to proper controls makes some of the results difficult to interpret.

No statistics accompany the survival and growth results for Fig 4A-B, especially for the claim "Parental cell lines also showed a slight yet discernible increase in PLX4032 sensitivity upon YAP/TAZ knockdown (Fig 4A and 4B)."

No reference is given for the micro-pattern experiments in Fig 7A-B.

The authors state "it has been shown that changes in mechanical stimuli and cytoskeleton tension directly affect YAP/TAZ localization in mammalian cells," yet it is not clear what alternative hypothesis is being ruled out in Fig 7A-D. Results are stated as both 'expected' and 'remarkable'.

It is unclear whether the reduced viability from CytoD (Fig 7E), blebbistatin (Fig 7E), or siTESK1 (Fig 8I) treatment is from the YAP down-regulation as claimed or by the inhibition of cytoskeletal dynamics, especially since significant toxicity is evident in WM3248 not exposed to PLX4032. A necessary control would be along the lines of using YAP5SA expression cells to show that YAP can rescue this cell death.

Figure 7H is not convincing. Immunofluorescence showing as few as 1 cell per condition, and without quantitation.

Statistical analysis and false discovery rates/corrections are not presented for the knockdown screen.

The result stated by the authors, "further confirming the importance of YAP/TAZ in PLX4032 resistance, LATS2 knockdown promoted the growth of resistant cells (Fig 8B)" is not supported by statistical analysis and does not appear to be statistically significant in the knockdown figure.

The authors write that "suppression of actin remodeling may be an efficient way to overcome BRAF inhibitor resistance." Efficient in what sense? Clinically? Is a clinical approach feasible?

Western blots of Fig 3D are not convincing and do not state that replicate experiments were performed.

Fig 7G-H are not convincing. Western blots without quantitation or replicates.

There are instances of error bars with no stated number of replicates.

1st Revision - authors' response

15 September 2015

Response to Referees' Comments

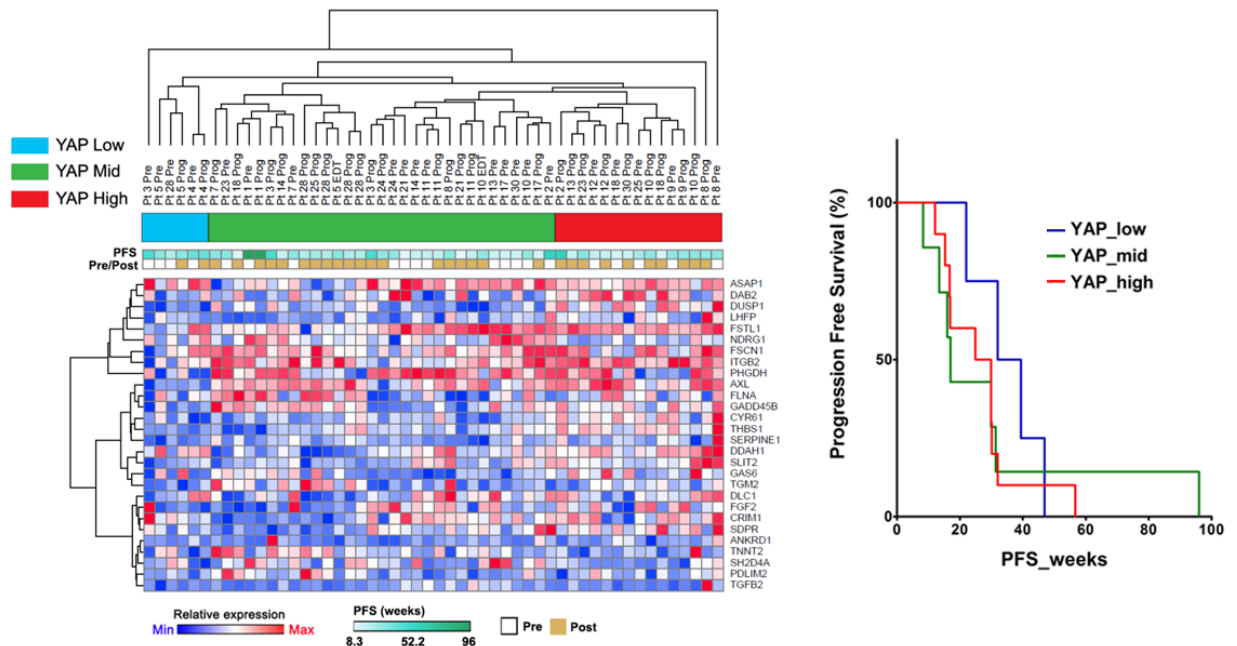
Referee 1.

1. The authors are just focusing on 3 YAP/TAZ targets upregulated in resistant cells. The authors should be more convincing by deriving YAP/TAZ-dependent gene signature in melanoma cells (even better if resistant vs. non resistant cells; part of the work on YAP/TAZ dependency has been already done in fig 6) and compare it public datasets to infer the effects on survival in patients treated with B-raf inhibitors.

We appreciate the insightful comments by the Referee, which have motivated additional experiments and deeper thinking. We have performed microarray analyses, comparing parental and resistant cells of both SKMEL28 and WM3248. The gene-set enrichment analysis (GSEA), comparing resistant SKMEL28 & WM3248 vs. parental SKMEL28 & WM3248, confirmed a significant enrichment of YAP-target gene signature in resistant cells. This result is presented in Fig 3I and Fig EV 2F. The microarray data revealed not only YAP/TAZ dependency of resistant cells but also altered expression of genes associated with actin cytoskeletal architecture (please see our response to the referee 2's comment 4).

We have also analyzed public datasets to address the effects of YAP/TAZ-signature on melanoma patient survival after BRAF inhibitor treatment. We obtained GSE50509 data deposited in Gene Expression Omnibus (GEO), which present expression profiles of 52 fresh-frozen melanoma tumor samples from 21 BRAF-mutant patients who received biopsy before and after treatment with dabrafenib or vemurafenib (Rizos et al., *Clin Cancer Res* 2014, 20, 1965-1977). Patient characteristics including progression free survival (PFS) on BRAF inhibitor therapy was obtained from the same article. We performed hierarchical clustering of these 52 tumor profiles using the

leading edge subset of YAP signature genes (28 genes) upregulated in resistant melanoma cells on GSEA shown in Fig EV2F. We classified the tumor samples into YAP low, YAP mid, and YAP high groups based on the mean expression level (log2) of YAP signature genes of each cluster. We also classified the patients according to YAP status of their tumors (YAP low patients, YAP low tumor in either pre and post biopsies; YAP high patients, YAP high tumor in either pre and post biopsies; YAP mid patients, the others). We found numerically shorter PFS of YAP mid/high patients compared with YAP low patients in Kaplan Meier survival analysis (PFS, 35.7 weeks for YAP low; 17 weeks for YAP mid; and 27.4 weeks for YAP high) (excluded Figure 1). However, statistical significance was not reached ($p=0.647$ by log rank test). Thus, we do not include the result in our revised manuscript. Further prospective clinical studies would be needed to define prognostic significance of YAP signature in BRAF inhibitor therapies.



Excluded Figure 1. Classification of clinical BRAF-mutant melanoma samples based on YAP signatures and its correlation with progression free survival on BRAF inhibitor therapy. The heatmap shows hierarchical clustering of 52 melanoma tumor samples with YAP signature genes. The Kaplan Meier plot shows progression free survival of YAP low, YAP mid and YAP high patients after BRAF inhibitor treatment.

2. They focus on YAP/TAZ and imply that this is involved in resistance. However it is unclear to me if this is really specific. Given the pivotal role of YAP in cell proliferation, one wonders if YAP would surpass resistance of everything. I do realize that this is somewhat a "philosophical" concern. However, some better controls are still due. It is unclear for me if the effect of YAP, TAZ or combined YAP/TAZ depletions is really stronger (in fold induction) in resistant vs non-resistant cells. Parental cells have not been examined. While I suspect that YAP/TAZ inactivation may have just the same effects, there may be a "window" of specificity using the partial/single YAP or TAZ knockdowns.

We understand the referee's concern. In the revised manuscript, we present additional evidence showing elevated sensitivity of resistant cells to YAP/TAZ depletion. To find "window" of specificity, we challenged variable doses of YAP/TAZ siRNAs to parental and resistant cells. Importantly, we observed significantly higher cell viability loss in resistant cells at multiple siRNA doses (Fig EV 3D). Resistant cells showed significant loss of cell viability even at low siRNA doses (1.25 and 2.5 nM), which did not affect the viability of parental cells. In addition, our results shown in Figs 4C and D, and EV3C indicate that proliferation (% BrdU+ cells) of resistant cells is more sensitive to YAP/TAZ depletion than that of parental cells. Therefore, we conclude that resistant cells exhibit increased dependency on (or addiction to) YAP/TAZ activity for their survival when compared with parental cells. It is likely that suppression of YAP/TAZ would be particularly efficient for overcoming drug resistance in cancer cells that are addicted to YAP/TAZ.

3. Figure 7G and H. The regulation of protein levels by transfected LATS is problematic. YAP is hardly changed at the total protein level, questioning the specificity of these results. Moreover the Hippo pathway is regulated by LATS phosphorylation and not by reduced protein levels. Oppositely, new evidence in the field suggests the opposite: LATS kinases are typically induced by the active YAP as a negative feedback. Overall we have no clue on what regulate LATS levels in these cells. The YAP overexpression and subsequent speculation are thus irrelevant if not misleading. Finally, while it is clear that the cytomechanics and F-actin control YAP/TAZ, work from several groups - including the original papers from S.Piccolo group - failed to reveal a functional role of YAP phosphorylation by LATS downstream of F-actin and Cofilin (addressed in Fig 8). There are no definitive answers and the intersections with the cytoskeleton occur at many levels. All this clearly requires a set of dedicated studies. For the time being, I suggest simply to remove these results; they can eventually keep it in discussion as a speculative point.

We fully agree with the referee. Both the interaction between the Hippo pathway and the cytomechanics/F-actin pathway and their relative contribution to YAP control are still being debated. Although we observed differences in total protein levels of LATS1 between parental and resistant melanoma cells, we were not able to determine whether the Hippo pathway activity in parental/resistant cells is different. In addition, it is not clear if altered actin cytoskeleton architecture in resistant cells influences the regulation of YAP/TAZ by the Hippo pathway. We think resolving these issues would be beyond the scope of this study. As recommended by the referee, we have removed Fig 7G and H of the previous version of our manuscript, which are potentially misleading. Instead, we have focused on confirming the finding that reorganization of actin cytoskeleton architecture is a key mediator of YAP/TAZ activation in resistant cells. First, we show that resistant cells exhibit remarkable changes in actin cytoskeletal organization (Fig. 2D, E, and F). Second, our new microarray analysis reveals changes in the expression levels of multiple actin cytoskeleton regulators (Fig. 2G; Fig EV1A and B). Moreover, YAP/TAZ activity is highly sensitive to the modulation of F-actin polymerization and actomyosin contraction in resistant cells (Figure 7C-F).

4. Figure 2: the b-catenin staining is inconclusive. I see nuclear staining as well in one panel. Please substitute with a different membrane marker. Or with simple bright-fields for cell area/shape.

We have substituted this result with a new experiment, using Alexa 594-conjugated wheat germ agglutinin (WGA) to mark cell boundaries (Fig 2B and C). As expected, we obtained the same result as our first measurement with β -catenin immunostaining, confirming enhanced spreading of resistant cells.

5. I can hardly take any serious conclusion from Fig. 3C.

We think this result is important, because it shows that YAP/TAZ nuclear enrichment and F-actin accumulation (Fig 2F) occurs on a similar timescale in response to PLX4032. To further validate this result, we have included quantifications of nuclear YAP/TAZ localization in both SKMEL28 and WM3248 cells after PLX4032 treatment (Figs 3D, EV2C and D). Nuclear YAP/TAZ localization peaks after 7 days of PLX4032 treatment in both cell lines.

6. siYAP/TAZ: figure 4 and all other panels. ALL the key experiments should be repeated with an independent pair of siRNAs and rescued with cDNA. The risk of offtarget effects is known.

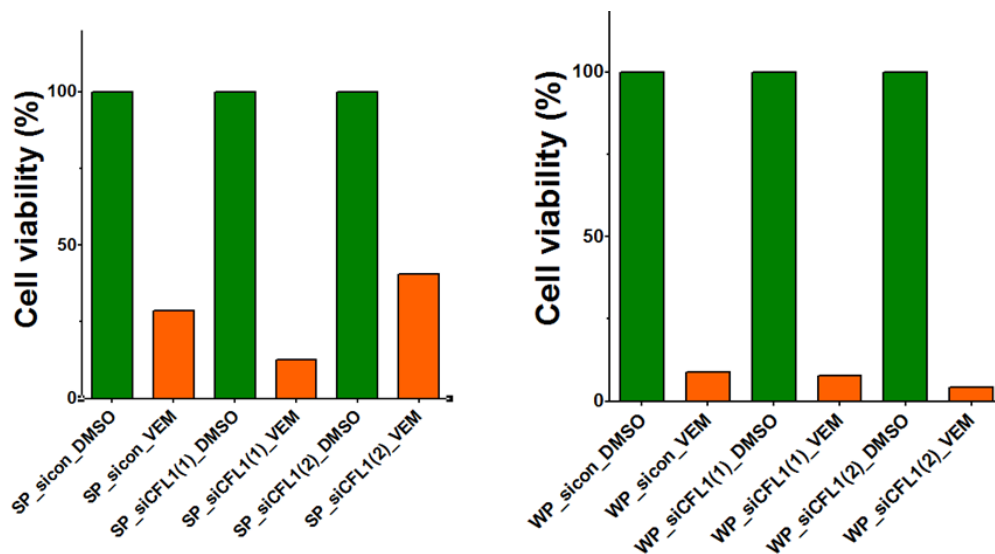
We agree with the referee. We have repeated key experiments with an independent pair of YAP and TAZ siRNAs, which were used in a published work (Dupont et al. *Nature* 2011, 474,179-183). Confirming our conclusion that resistant cells depend on YAP/TAZ activity for survival, YAP/TAZ knockdown using the second siRNA pair have reproduced similar results from PLX4032 dose-response, viability time course, and BrdU incorporation in both SKMEL28 and WM3248 cells (Fig EV3A-C, and E). In addition, we show that loss of cell viability upon YAP/TAZ knockdown in resistant cells can be rescued by siRNA-resistant YAP-5SA cDNA (Fig EV3F and G).

7. The myc connection is quite intriguing and novel. Can Myc rescue siYAP/TAZ (re-establishing resistance)?

To address this suggestion, we have performed several new experiments. First, we confirmed the strong influence of YAP/TAZ on c-MYC expression levels in resistant cells. Our western blot (Figure 4E), immunofluorescence analyses (Fig EV4A), and qRT-PCR assay (Fig EV4B) showed that YAP/TAZ knockdown causes a substantial reduction of c-MYC protein and mRNA levels. Microarray data also showed that the expression of MYC-signature genes decreases in resistant cells depleted of YAP/TAZ (Fig 6F), indicating that c-MYC target gene expression is dependent on YAP/TAZ in resistant cells. Moreover, we observed a decrease in cell viability after c-MYC knockdown in resistant SKMEL28 cells (Fig 4G). However, in accord with the low expression level of c-MYC in resistant WM3248 cells (Fig 1F), c-MYC knockdown did not affect the viability of resistant WM3248 cells, suggesting that the contribution of c-MYC in cell survival is negligible in resistant WM3248 (Fig EV 4E). From this result, we speculate that the impact of c-MYC upregulation on BRAF inhibitor resistance is cellular context dependent. In addition, c-MYC overexpression failed to rescue cell viability loss caused by YAP/TAZ knockdown in both resistant SKMEL28 and resistant WM3248 (fig EV4F and G). It is possible that c-MYC alone may not be sufficient for rescuing YAP/TAZ depletion because YAP/TAZ in resistant cells support multiple cell survival pathways involving E2F, AKT and EGFR. We have included explanation for our data on c-MYC in the revised manuscript.

8. Fig. 8. One experiment to attempt is whether knockdown of Cofilin does the opposite of TESK in SKMEL and WM cells (i.e. induce resistance in non-resistant/parental cells). It may be asking for too much, but if works it would complete the panel and make a strong point on F-actin.

This is an interesting suggestion. We have analyzed changes in PLX4032 sensitivity of parental SKMEL28/WM3248 cells upon Cofilin1 knockdown. As shown below, Cofilin1 knockdown was not sufficient to induce PLX4032 resistance in parental cells. Although Cofilin1 is known to play a key role in actin dynamics, our microarray data comparing parental and resistant cells revealed that the architectural change in the actin cytoskeleton may be ascribed to altered expression of a group of genes associated with the actin cytoskeleton (Figs 2G, Fig EV1A and B). Besides, Cofilin and phospho-Cofilin levels did not appear to be changed consistently in resistant cells (Fig EV5D). Thus, Cofilin may not be a major driver of actin cytoskeleton remodeling and PLX4032 resistance.



Excluded Figure 2. Cell viability assay of parental SKMEL28 and WM3248 cells upon Cofilin1 knockdown. Cells were transfected with either control (siCon) or two independent cofilin1 siRNAs, siCFL1(1) and siCFL1(2) for 72 hr, and then treated with 2 μ M PLX4032 or DMSO for additional 72 hr. Relative cell viability, compared to DMSO control with control siRNA transfection, was measured by CCK8 assay.

9. Page 5. 2nd line, the ref is Zhao et al 2012.

We thank the referee for this comment. We have changed the citation.

10. Page 5 8XGTIIC-luciferase needs a reference. Why not call this a TEAD reporter? Repeat the color legend in main figure Fig 8G.

We have revised the sentence: “YAP/TAZ-responsive TEAD reporter, 8XGTIIC-luciferase (Dupont et al, 2011)”. We have included color legend in Fig 8F (previously Fig 8G).

11. Why the effect of TESK knockdown is so weak in WM cells?

As shown in Fig 8D, TESK1 knockdown causes a significant reduction of the survival of resistant SKMEL28 and WM3248 cells. Nuclear YAP/TAZ enrichment is also reduced by TESK1 knockdown (Fig 8E and F). Moreover, we show that cell viability loss caused by TESK1 knockdown can be rescued by YAP5SA (Fig EV5B). However, as indicated by the referee, the percentage of cells showing nuclear YAP is relatively higher in resistant WM3248 cells even after TESK1 knockdown. We speculate that the observed weaker reduction is ascribed to higher baseline nuclear YAP/TAZ localization in resistant WM3248 cells. However, we think the weak reduction in nuclear YAP/TAZ localization may be sufficiently lethal for resistant WM3248 cells, possibly because resistant WM3248 cells are addicted to YAP/TAZ.

12. Discussion must be greatly simplified while remaining on the merit of the MS. For example: The first paragraph is useless. Discussion on existing small molecules can be deleted. They were not tested in this context.

We appreciate these insightful comments, and we have re-written the discussion to conform to the referee’s statement. We have reduced content of the first paragraph, and removed the description on small molecules from the discussion section.

13. The discussion on Mori et al: as discussed I do not believe it is pertinent. They propose a transcription-independent mechanism in that paper. Is this what they imply for YAP/TAZ here? I doubt it, as they use CTGF as transcriptional read out. How c-Myc is regulated here remains undefined.

We agree with the referee’s comment. We have removed a discussion on Mori et al.’s paper. In our data, c-MYC mRNA level was decreased upon YAP/TAZ depletion (Fig EV4B), which is distant from Mori et al’s point.

14. The discussion on Feng et al makes sense to the general reader only if it is clarified somewhere that Feng concludes that the cytoskeletal and Hippo/LATS cascades are distinct

We have revised our sentence as “a recent study demonstrated that activating mutation in GNAQ gene stimulates YAP through the promotion of actin polymerization, independent of the Hippo pathway, in uveal melanoma (Feng et al, 2014)” to clarify that Feng et al. concludes that the cytoskeletal and Hippo/LATS cascades are distinct.

Referee 2.

1. Previous studies have implicated YAP/TAZ in resistance to RAF inhibitors (Lin et al 2015 Nature Genetics). While this study is noted, there is an incremental advance provided by the current study.

We appreciate the referee’s comments on our work. Lin et al paper showed that YAP can provide drug resistance and the result was thoroughly confirmed by clinical samples. However, they did not show the mechanism of aberrant YAP activation in resistant tumor samples. Our study demonstrates that (i) BRAF inhibitor PLX4032 induces changes in actin cytoskeletal architecture and (ii) the changes are responsible for aberrant YAP/TAZ activation, which promotes resistance to PLX4032.

2. Other studies have implicated a role for extracellular matrix/integrin signaling in resistance to RAF inhibitors and changes in cell morphology/actin cytoskeleton (e.g. Hirati et al. 2015 Cancer Cell; Shao et al 2012 Cell Death Diff). Thus, the notion that actin cytoskeletal remodeling is linked to resistance is not new.

We agree with the referee. However, the indicated articles mainly focus on the FAK/Src pathway and the interaction between ECM and cancer cells. We propose YAP/TAZ activation by actin cytoskeletal remodeling within cancer cells as a novel mechanism of adaptive drug resistance. Our results prompt the need for further studies on the relationship between alterations in the extracellular matrix and intracellular cytoskeletal remodeling after drug treatment. A recent study identified a link between FAK/Src/PI3K pathway in the regulation of the Hippo signaling (Kim & Gumbiner, *J Cell Biol* 2015, 210, 503-515). Thus, it is possible that YAP/TAZ is a key intracellular effector of the drug-tolerant microenvironments with high integrin β 1/FAK signaling which were suggested by Hirati et al.

3. Multiple independent siRNAs need to be utilized per target (YAP/TAZ) to eliminate concerns about off-target effects.

We have repeated key experiments with an independent pair of YAP and TAZ siRNAs, which were used in a published work (Dupont et al. *Nature* 2011, 474,179-183). Confirming our conclusion that resistant cells depend on YAP/TAZ activity for survival, YAP/TAZ knockdown using the second siRNA pair have reproduced similar results from PLX4032 dose-response, viability time course, and BrdU incorporation in both SKMEL28 and WM3248 cells (Fig EV3A-C, and E). In addition, we show that loss of cell viability upon YAP/TAZ knockdown in resistant cells can be rescued by siRNA-resistant YAP-5SA cDNA (Fig EV3F and G).

4. What mediates the actin reorganization in the vemurafenib resistant cells is unclear. TESK1 is not upregulated in the resistant SKMEL28 cells. The majority of the data are shown with resistant WM3248.

We appreciate the referee's comment on this critical issue. Changes in actin dynamics can occur within a few minutes. However, PLX4032-induced actin reorganization takes over a week, and the changes are stably maintained. Thus, we suspected that epigenetic mechanisms controlling the expression of genes associated with actin cytoskeletal architecture might be involved in the actin reorganization induced by PLX4032. We have performed additional microarray analysis comparing resistant SKMEL28 & WM3248 vs. parental SKMEL28 & WM3248. We found 829 significantly altered genes in both resistant cells (Fig EV1A), and performed gene ontology analysis with the hits. Remarkably, multiple actin cytoskeleton-related functional categories were enriched on significantly altered genes in resistant cells (Fig EV1B). Fig 2G of the revised manuscript presents 40 actin-associated genes showing consistent changes in resistant cells (both SKMEL28 and WM3248). Therefore, it would be reasonable to think that changes in global expression levels of actin-associated genes collectively reorganize the actin cytoskeleton in response to PLX4032 treatment. As indicated by the referee, correlation between TESK1 mRNA expression levels and drug resistance is inconsistent. Besides, we have added a western blot result showing that neither TESK1 protein nor phospho-Cofilin levels in resistant cells are consistently increased (Fig EV 5D). These results suggest that changes in TESK1 expression levels may not be causally linked to the establishment of PLX4032 resistance. Nevertheless, we think TESK1 serves as an important vulnerable point of YAP/TAZ activated resistant melanoma cells, because TESK1 depletion effectively suppresses both YAP/TAZ activity and resistant cell viability.

5. The use of the term adaptive resistance is unclear in the context of this manuscript.

We suggest YAP/TAZ activation in response to BRAF inhibitor is "adaptive" resistance mechanism because it does not act innately (immediate after drug treatment) and it does not seem to involve acquisition of additional mutations affecting YAP/TAZ, which may require long-term drug treatment (several months). The observation that both actin reorganization and YAP/TAZ nuclear enrichment after PLX4032 treatment are not restricted to a minor subpopulation of cells, and the changes occurs in a short period of time (1~2 weeks) collectively suggest that YAP/TAZ-dependent drug resistance is an epigenetic adaptive response of melanoma cells to the BRAF inhibitor. We conclude that epigenetic changes in transcriptional programs affecting actin organization and YAP/TAZ targets underlie the observed drug resistance in SKMEL28 and WM3248 cells.

6. TESK1 as a therapeutic target is unclear. The frequency of TESK1 alterations in human samples is unclear.

We think TESK1 or other actin dynamics regulators could be therapeutic targets, because cancer cells are subjected to continuous actin cytoskeletal remodeling and actin organization can be modulated by targeting actin regulators. However, we think further validation using *in vivo* models is necessary for claiming that TESK1 is a good target for overcoming BRAF-inhibitor resistance in melanoma. According to the referee's comment, we surveyed TCGA melanoma database using cBioportal for Cancer Genomics, and found TESK1 alterations (mutation, amplification, or mRNA upregulation) in 5% of evaluated tumor samples (Fig EV5E). Further studies are needed to characterize TESK1 alterations in terms of BRAF inhibitor resistance and YAP/TAZ activation.

Referee 3.

1. There are a number of discrepancies between the two cell lines, in some cases one cell line fits the model better and in other cases where this is reversed. While SKMEL28 is generally consistent with the author's claims when both cell lines are presented, it is unclear why only WM3248 is presented in others. There are difficulties reconciling these differences, and lack of acknowledgement and discussion of the discrepancies by the authors. Additional cell lines should be evaluated to strengthen the claims.

We understand this concern. In our initial submission, we omitted some experiments simply because the growth of resistant SKMEL28 cells was slower than resistant WM3248. We have repeated all major experiments using SKMEL28 cells, and have acquired data that are consistent with our conclusions (Fig 2F, Fig 4E, Fig 6, Fig 8G, Fig EV 2A-D, Fig EV 5A). Because validation on additional cell lines can substantially delay our revision, we have instead focused on confirming our conclusions using SKMEL28 and WM3248.

2. Knockdown of YAP in WM3248 resistant cells do not completely restore sensitivity in the WM3248 cell line (Fig 4A), with the highest concentration only decreasing relative survival to 50%. Yet, the manuscript text generalizes across the two cell lines "a remarkable restoration of sensitivity."

We agree that "remarkable restoration" is not an appropriate phrase in describing WM3248 dose response after YAP/TAZ knockdown. We have gone through the manuscript to modify overstatement or inappropriate generalization. Although dose-response curve of resistant WM3248 was not shifted dramatically, their proliferation was significantly decreased after YAP/TAZ depletion. The difference between SKMEL28 and WM3248 cells in the sensitivity to YAP/TAZ knockdown might be ascribed to distinct genetic/epigenetic contexts of the two cell lines. We have included a new sentence: "Restoration of PLX4032 sensitivity after YAP/TAZ knockdown was more prominent in resistant SKMEL28 cells than in resistant WM3248 cells."

3. Across the two lines, the role of MYC is unclear. While resistance and active YAP5SA expression prevents PLX4032 mediated down-regulation of MYC, MYC levels also decrease with establishment of resistance in WM3248 cells (to levels even lower than in PLX4032-treated SKMEL28 cells) (Fig 1F).

We thank the referee for this sharp criticism. What we have discovered is that c-MYC expression is BRAF-dependent in parental SKMEL28/WM3248 cells, whereas c-MYC expression is promoted by YAP/TAZ activity in resistant SKMEL28/WM3248 cells (Fig 1F and Fig 4E). Our microarray data showing downregulation of MYC-signature genes upon YAP/TAZ knockdown indicates that, at least, c-MYC target gene expression is dependent on YAP/TAZ in resistant cells. Moreover, we observed a decrease in cell viability after c-MYC knockdown in resistant SKMEL28 cells (Fig 4G). However, in accord with the low expression level of c-MYC in resistant WM3248 cells, c-MYC knockdown did not affect the viability of resistant WM3248 cells, suggesting that the contribution of c-MYC in cell survival is negligible in WM3248 (Fig EV 4E). From this result, we speculate that the impact of c-MYC upregulation on BRAF inhibitor resistance is cellular context dependent. In addition, c-MYC overexpression failed to rescue cell viability loss caused by YAP/TAZ knockdown in both resistant SKMEL28 and resistant WM3248 (fig EV4F and G). It is possible that c-MYC alone may not be sufficient for rescuing YAP/TAZ depletion because YAP/TAZ in resistant cells support multiple cell survival pathways involving E2F, AKT, and EGFR. We have included explanation for our data on c-MYC in the revised manuscript.

4. There is no explanation as to why the results in Fig 4E are shown for only WM3248 cells. Fig 4E results are stated in the text with no interpretation or follow-up. What is the importance of these findings, especially in light of the low MYC levels in resistant WM3248 cells (Fig 1F)?

We have performed western blot analysis for resistant SKMEL28. As shown in revised Fig 4E, downregulation of EGFR, c-MYC and phospho-AKT is also observed in resistant SKMEL28 cells after YAP/TAZ knockdown. To test if EGFR and AKT contribute to YAP/TAZ-dependent cell survival, we have performed cell viability assay with/without the treatment of EGFR and AKT inhibitors (Figs 4F and EV4C). AKT inhibitor, MK-2206, solely reduced cell viability in resistant SKMEL28 and WM3248 cells. Moreover, combination of EGFR, AKT, BRAF inhibitions resulted in additional loss of cell viability when compare with AKT inhibitor alone. Taken together with c-MYC knockdown phenotype (Fig 4G) and microarray data (Fig 6), these observations suggest that multiple oncogenic pathways supported by YAP/TAZ activity contribute to the survival of resistant cells.

5. The microarray gene expression experiment in Fig 6 is lacking the use of a parental control line to demonstrate that the observed enrichment results are only present in resistant cells. It is unclear why the WM3248 line was chosen for these studies. Profiling both cell lines would strengthen the arguments. The focus on MYC in this section further raises the question on the observed substantial decrease in MYC protein levels in resistant WM3248 but not resistant SKMEL28 (1F).

To address this criticism, we have performed additional microarray analyses (Fig 6, Fig EV 1A and B, Fig 2G, Fig 3I, and Fig EV2F). We have identified (i) genes (and signature) whose expression levels change significantly in resistance cells (SKMEL28 Resistant vs. Parental; WM3248 Resistant vs. Parental), and (ii) genes (and signature) whose expression levels significantly change in response to YAP/TAZ knockdown (resistant SKMEL28 cont si vs. YAP/TAZ si; resistant WM3248 cont si vs. YAP/TAZ si). We observed significant enrichment of YAP signature on resistant cells compared with parental cells (Fig 3I). We found similar results in resistant SKMEL28 cells upon YAP/TAZ siRNA knockdown as in resistant WM3248 cells (Fig 6): downregulation of cell cycle pathway, E2F1, EGFR and c-MYC signatures. YAP/TAZ signature is activated in resistance cells (Fig 3I) and YAP/TAZ knockdown has clear impact on resistant cell survival, not on parental cell survival (Fig 4A, Fig EV3D).

6. The author's state "As expected, the reduction of cell viability after TESK1 knockdown was higher in resistant SKMEL28 and WM3248 cells than the reduction in parental cells (Fig 8I)" but in this discussion they do not address the fact that in the WM3248 parental cells, the reduction in viability by TESK1 knockdown was very large, which is in stark contrast to the effect in SKMEL28 parental cells. Furthermore, the results of Fig 8C have a reversal in regards to which cell line shows a difference between parental and resistant sublines, this time in terms of TESK1 expression levels, which is difficult to reconcile with the opposing trends seen in 8I. Why is the biomarker pattern not predictive of the knockdown response?

We understand the referee's concern. It is still not clear why parental WM3248 cells are so sensitive to TESK1 knockdown in the regular culture media of WM3248 containing 2 % FBS. Interestingly, however, we have found that the survival of parental WM3248 cells depleted of TESK1 can be substantially improved by adding 10 % FBS (Fig 8D). In this condition, we were able to clearly observe higher sensitivity of resistant WM3248 cells to TESK1 knockdown compared with parental cells. Moreover, both nuclear YAP/TAZ enrichment and YAP target gene expression were reduced by TESK1 knockdown in resistant cells (Fig 8E-G). However, it is unlikely that TESK1 upregulation is causally linked to the development of BRAF inhibitor resistance. Originally, we speculated that alteration in TESK1 expression or activity could underlie PLX4032-induced actin reorganization. Based on our new microarray analysis (Fig 2G, Fig EV1A-B), we now think that global changes in the expression levels of genes associated with actin cytoskeletal architecture underlie actin reorganization in response to PLX4032 treatment. We have performed microarray analysis comparing resistant SKMEL28 & WM3248 vs. parental SKMEL28 & WM3248. We found 829 significantly altered genes in both resistant cells (Fig EV1A), and performed gene ontology analysis with the hits. Remarkably, multiple actin cytoskeleton-related functional categories were enriched on significantly altered genes in resistant cells (Fig EV1B). Fig 2G of the revised manuscript presents 40 actin-associated genes showing consistent changes in resistant cells (both

SKMEL28 and WM3248). Therefore, it would be reasonable to think that changes in global expression levels of actin-associated genes collectively reorganize the actin cytoskeleton in response to PLX4032 treatment. As indicated by the referee, correlation between TESK1 mRNA expression levels and drug resistance is inconsistent. Besides, we have added a western blot result showing that neither TESK1 protein nor phospho-Cofilin levels in resistant cells are consistently increased (Fig EV 5D). Apparently, TESK1 is not a biomarker for YAP-dependent BRAF inhibitor resistance. Nevertheless, we think TESK1 serves as an important vulnerable point of YAP/TAZ activated resistant melanoma cells, because TESK1 depletion effectively suppresses both YAP/TAZ activity and resistant cell viability.

7. The authors state that "Survived resistant clones were maintained in the presence of 2 μ M PLX4032." However, no details are given regarding the timing of drug removal and subsequent exposure when resistant cells were used in experiments that compared with and without PLX4032 conditions. These aspects are critical due to rebound effects and kinetics of drug removal and exposure, especially for pERK. Signs of such effects are seen in the increase growth of one line when low doses of PLX4032 are present (Fig 1A, 4A). The absence of such detail, and explanation of consideration given to proper controls makes some of the results difficult to interpret.

We have continuously applied 2 μ M PLX4032 to resistance cells for all maintenance procedures, because removal of PLX4032 results in loss of resistance as indicated by a recent study (Sun et al., Nature 2014, 508, 118-122). For all experiments, cells were seeded on a dish (or a chamber) without PLX4032, and after 24 hour incubation, PLX4032 or DMSO was applied to cells for the indicated time courses described in each figure legend. We have revised our figure legends to describe the timing of drug removal and subsequent exposure.

8. No statistics accompany the survival and growth results for Fig 4A-B, especially for the claim "Parental cell lines also showed a slight yet discernible increase in PLX4032 sensitivity upon YAP/TAZ knockdown (Fig 4A and 4B)."

For Fig 4A and Fig EV3A, we have performed statistical analysis comparing IC50 of cells upon YAP/TAZ siRNA and control siRNA transfection. Sigmoidal dose-response curves were fitted to data and IC50 values of cells with control siRNA and YAP/TAZ siRNA knockdown were compared using extra sum-of-squares F test by GraphPad Prism. Resistant SKMEL28 and WM3248 cells showed significant decreases in cell viability in response to YAP/TAZ knockdown. Parental cells also showed a slight increase in PLX4032 sensitivity upon YAP/TAZ knockdown, but only WM3248 cells transfected with YAP/TAZ siRNA set #1 showed statistical significance (Fig 4A). We have revised the manuscript according to our statistical analysis.

9. No reference is given for the micro-pattern experiments in Fig 7A-B.

We purchased micropatterned coverslips used in this study from CYTOOchips™ (<https://cytoo.com/>). We have added a sentence in method section as "All micropatterns used in this study were produced by CYTOO (<http://www.cytoo.com/>)", and also added reference as "They M (2010) Micropatterning as a tool to decipher cell morphogenesis and functions. Journal of cell science 123: 4201-4213".

10. The authors state "it has been shown that changes in mechanical stimuli and cytoskeleton tension directly affect YAP/TAZ localization in mammalian cells," yet it is not clear what alternative hypothesis is being ruled out in Fig 7A-D. Results are stated as both 'expected' and 'remarkable'.

As described in the introduction, YAP/TAZ activity in mammalian cells is influenced by various signaling inputs including GPCR, WNT signaling, EGF, mechanical stress, and cell-cell/cell-matrix contact. Because we observed reproducible changes in the actin cytoskeleton in PLX4032-resistant cells compared with parental cells, we examined whether actin reorganization is a major cause for YAP/TAZ activation in resistant cells. Experiments shown in Fig 7A-D demonstrate that actin polymerization, actin-myosin contraction, and mechanical stress (micropattern) are potent regulators of YAP/TAZ localization in resistant cells. One important question (alternative hypothesis) is whether changes in the Hippo pathway activity are also involved in YAP/TAZ regulation of melanoma cells. Both the interaction between the Hippo pathway and the cytomechanics/F-actin pathway and their relative contribution to YAP control are still being debated. Thus, it requires

additional sets of dedicated studies to resolve the issue, which might be beyond the scope of this study. As suggested by the referee 1, we have removed experimental results involving the Hippo pathway from Fig 7, and have completed our study by demonstrating the impact of the actin cytoskeleton in YAP/TAZ-dependent PLX4032 resistant. We have included a comment in the discussion that alternative hypotheses remain to be tested.

11. It is unclear whether the reduced viability from CytoD (Fig 7E), blebbistatin (Fig 7E), or siTESK1 (Fig 8I) treatment is from the YAP down-regulation as claimed or by the inhibition of cytoskeletal dynamics, especially since significant toxicity is evident in WM3248 not exposed to PLX4032. A necessary control would be along the lines of using YAP5SA expression cells to show that YAP can rescue this cell death.

This is an excellent suggestion, and we address it specifically in the revised manuscript. We have performed the suggested rescue experiment using YAP-5SA vector. As expected, YAP-5SA transfection significantly rescued cell viability loss caused by CytoD+PLX4032 or Bleb+PLX4032 in resistant SKMEL28 and WM3248 cells (Fig 7G). Cell viability loss caused by TESK1 knockdown was also rescued by YAP-5SA (Fig EV5D). These results support involvement of YAP in cell viability suppression in response to cytochalasin D, Blebbistatin or TESK1 knockdown.

12. Figure 7H is not convincing. Immunofluorescence showing as few as 1 cell per condition, and without quantitation.

As discussed above (comment 10), we have removed Fig 7H.

13. Statistical analysis and false discovery rates/corrections are not presented for the knockdown screen. The result stated by the authors, "further confirming the importance of YAP/TAZ in PLX4032 resistance, LATS2 knockdown promoted the growth of resistant cells (Fig 8B)" is not supported by statistical analysis and does not appear to be statistically significant in the knockdown figure.

In our revised manuscript, we present the knockdown screen data using z-score method, which is a common practice in RNAi library screening (Fig 8B). We calculated z-score of normalized cell viability of target siRNAs for each replicates, and target siRNAs with Z scores < -2 in both replicates were considered as significant synthetic lethal hits. We also considered genes with Z score > 2 in both replicates as growth promoting hits. As a result, LATS2 was included in growth promoting hits, suggesting the importance of YAP/TAZ in PLX4032 resistance. We added Z-score data of each two replicate and screening hits in Figure 8B. In our opinion, RNAi library screen data are not suitable for a statistical test followed by multiple comparison correction when the screen is done in duplicates.

14. Western blots of Fig 3D are not convincing and do not state that replicate experiments were performed. Fig 7G-H are not convincing. Western blots without quantitation or replicates.

Fig 3D has been relabeled as Fig 3E. We have repeated the experiment, and quantitation of the results are shown in Fig 3F (total three biological replicates). TAZ level in resistant WM3248 was not compared due to its low expression level. As discussed above (comment 10), we have removed Fig 7G and H.

16. The authors write that "suppression of actin remodeling may be an efficient way to overcome BRAF inhibitor resistance." Efficient in what sense? Clinically? Is a clinical approach feasible?

We thank the referee for this question (or healthy skepticism). We found that the survival of both SKMEL28 and WM3248 cells resistant to PLX4032 is highly dependent on F-actin polymerization and actomyosin contractility. In fact, clinical application of drugs that disrupt global actin dynamics may not be feasible because the actin cytoskeleton plays a wide variety of roles in normal cells. However, we think specific actin dynamics regulators including TESK1 could be good therapeutic targets. For example, inhibitors of LIMK2, a kinase related to TESK1, are under clinical trial. Of course, we understand that we should be careful about suggesting clinical application of our findings because our study does not include any in vivo data. Thus, we have gone through the manuscript to

modify overstatement, and revised the sentence as “suppression of actin remodeling is a candidate strategy to overcome BRAF inhibitor resistance”.

17. There are instances of error bars with no stated number of replicates.

According to the referee’s comment, we have included replicate numbers for all error bars.

2nd Editorial Decision

23 October 2015

Thanks a lot for submitting your revised manuscript to The EMBO Journal. I am very sorry for the delay in getting back to you with a decision, but I have now received the comments from referees #1 and 3.

As you can see below, both referees appreciate that the added data has strengthen the manuscript. Referee #1 is happy with the revised version as is while referee #3 still raises some issues with the revised version. I have discussed these issues further with referee #1 and we both agree that the remaining concerns can for the most part be addressed with appropriate text changes. Lets discuss further on how to resolve these last issues. You can use the link below to submit the revised manuscript

Thank you for the opportunity to consider your work for publication. I look forward to seeing the final version.

REFEREE REPORTS

Referee #1:

This is a very interesting paper; all the concerns have been addressed.

Referee #3:

The authors characterize a component of an interesting BRAF inhibitor resistance mechanism in melanoma in which actin cytoskeleton remodeling promotes YAP/TAZ nuclear localization and activity. The strength of the study lies in that it further characterizes a recently appreciated connection between these two mechanisms in melanoma treatment resistance. While the evidence presented in the paper does support this main conclusion, there remain inconsistencies, lack of complete controls, and results involving only modest changes.

Major points:

It remains of concern that only two cell lines were tested, and that for two key results the two cell lines used do not show consistent results.

Referring to inhibition of actin remodeling (abstract) and TESK1 targeting (discussion) as a ****promising**** synthetic lethal strategy are overstatements primarily because the two cell lines tested are not consistent in these regards.

For actin remodeling, Figure 7E does not show a synergistic (synthetic lethal) effect for the WM3248 cell line. Furthermore, a more formal synthetic lethal finding would require a comparison to the parental cell lines, as the authors did do in contrast for the Figure 8 studies. The statistical comparison (line and asterisk) shown in 7E is misleading, especially for the WM3248 line, since it does not incorporate the results from the other controls performed (+/- CytoD or Blebb in the absence of PLX). With all of this in mind, it is not clear why only the WM3248 cells were used in 7F. The associated text in the results section needs to convey these aspects.

For TESK1 knock down (Figure 8), the WM3248 cell line shows only mild sensitivity, and not under all culturing condition tested. The inconsistency between cell lines, and the sensitivity of the

results to the culture conditions, suggest that highly plastic melanoma cells could evolve adaptive resistant mechanisms to the strategy proposed. From this perspective, it is difficult to conclude that TESK targeting is a promising approach. For the same reasons, it is overstatement to say that TESK1 knockdown exerted a profound effect.

It cannot be claimed that YAP/TAZ knock down restores sensitivity to BRAF inhibition, as is done in the corresponding results sections bold subtitle, b/c this is not a consistent result across the two cell lines. It can be more reasonably claimed that resistant cells have higher dependency on YAP/TAZ. The results of Fig 4 overall suggest that resistant cells are more sensitive to YAP/TAZ knockdown, but do not support a true synergy/synthetic lethality with BRAF inhibition (see Fig 4D, individual and combined treatments with siYT and PLX are at best additive and mainly due to siYT). In regards to survival, what are the effects of siYT on the resistant cells (independent of PLX)? Fig 4A does not convey what is the effect on survival due to introduction of the siYT since the data in this panel is normalized.

Experiments using other targeted therapies shown in Figures 4E,F, and G needs to be performed on parental cell lines as a control for specificity to the resistant lines. Experiments in Fig 4F and EV4C are missing the PLX plus Erlotinib control. The authors do not comment on the finding that neither EGFR nor AKT fully explain the BRAF inhibitor resistance since treatment with corresponding EGFR and/or AKT inhibitors only partially kills the cells. Most of the reduction in cell viability is through MK-2206 (AKTi) and the addition of the other drugs does not add much effect. As was done for MYC, qualifications of these results need to be added in the results and discussion sections: 'Our finding suggests that YAP may serve as an important regulator of EGFR and AKT pathway activity in BRAF inhibitor resistance models, but that additional pathways are likely also involved in mediating resistance to BRAF inhibition (Fig 4F).' 'MK-2206 **partially** suppressed resistant cell survival, and combination with Erlotinib and PLX4032 resulted in **only slightly** higher suppression of cell viability. **No treatment strongly suppressed cell viability** We also depleted c-MYC expression by two independent c-MYC siRNAs (Fig EV4D), and observed a **slight** suppression of cell viability**,** but only in the **resistant SKMEL28 cells (Fig 4G).**'

Likewise, Figure 7 is difficult to adequately interpret due to a lack of parental controls. In contrast, Figure 8 does include parental controls. The authors state that only WM3248 shows sensitivity to Blebbistatin and CytoD, but in Fig 7G (as opposed to Fig 7E) SKMEL28 shows some sensitivity as well.

Minor points:

Results for Figure 3A-B need additional replicates and error bars, as done in the other panels in the figure.

The authors do not comment on why increased serum levels alter the WM3248 parental and resistant lines differential response to siTESK1 knockdown, nor do they explain their reasoning behind testing higher serum levels.

The GSEA results shown in Fig 3I are of borderline significance (NES = 1.4) and need to be acknowledged as such. Only one gene set is shown - the full GSEA results need to be shown in Table S3 for comparison, as done for the other enrichment analyses. The enrichment scores and statistical test values for 3I need to be stated in the legend.

The statement in the abstract that "Knockdown of YAP/TAZ overcomes PLX4032 resistance" needs to be qualified since this would not be true of all versions of BRAF inhibitor resistance (e.g. NRAS mutation, BRAF alternative splicing). Also on the second line of page 7.

YAP/TAZ nuclear localization does not peak at 7 days for WM3248 as stated by the authors (Figure 3C and 3D)

In Fig EV5C, TESK1 mRNA is several fold higher in WM3248 resistant cells than parental WM3248 and SKMEL28. Yet, the western blot in Fig EV5D does not show higher protein levels. This disconnect between mRNA and protein, combined with the inconsistent results seen between cell lines, indicate that a Western blot is needed to confirm reduction of TESK1 protein levels upon

TESK1 knockdown.

For Figure 8B, the authors used a z-score approach combined with an arbitrary threshold to filter for candidates. The rationale stated in their response to reviewer comments for not using a statistical test with multiple hypothesis correction was that "RNAi library screen data are not suitable for a statistical test followed by multiple hypothesis correction when the screen is done in duplicates." This statement is unclear. Consultation with a statistician will identify a statistical approach that can appropriately handle the replicate structure of the experiment.

There were no legends for the supplemental figures.

2nd Revision - authors' response

06 November 2015

(Underline indicates new experimental results.)

Referee 3

- Major points

1. It remains of concern that only two cell lines were tested, and that for two key results the two cell lines used do not show consistent results.

Additional cell lines would further strengthen our conclusion. However, validation on additional cell lines will substantially delay our revision, and we think our core findings are consistent between the two cell lines used. First, both SKMEL28 and WM3248 undergo actin remodeling upon PLX4032 treatment, and exhibit increased YAP/TAZ activity after PLX4032-resistance establishment.

Moreover, the viability of both resistant SKMEL28 and resistant WM3248 cells was significantly decreased by YAP/TAZ knockdown or inhibition of actin polymerization/contractility. The fact that SKMEL28 and WM3248 cells show somewhat different sensitivity to YAP/TAZ, c-Myc or TESK1 knockdown is not surprising because the two resistant cell lines' gene expression profiles are not identical although both of them are addicted to YAP/TAZ. In our final manuscript, we clearly indicate the differences between the two cell lines to avoid inappropriate generalization.

2. Referring to inhibition of actin remodeling (abstract) and TESK1 targeting (discussion) as a ****promising**** synthetic lethal strategy are overstatements primarily because the two cell lines tested are not consistent in these regards.

We agree that "promising" may be an overstatement. We have removed the word in the abstract and the discussion.

3. For actin remodeling, Figure 7E does not show a synergistic (synthetic lethal) effect for the WM3248 cell line. Furthermore, a more formal synthetic lethal finding would require a comparison to the parental cell lines, as the authors did do in contrast for the Figure 8 studies. The statistical comparison (line and asterisk) shown in 7E is misleading, especially for the WM3248 line, since it does not incorporate the results from the other controls performed (+/- CytoD or Blebb in the absence of PLX). With all of this in mind, it is not clear why only the WM3248 cells were used in 7F. The associated text in the results section needs to convey these aspects.

We appreciate the Referee's comment on this issue. Indeed, CytoD or Blebb alone showed substantial toxicity in WM3248 cells. Although PLX4032 treatment consistently caused additional viability loss, the change was not statistically significant. In our final manuscript, we address this issue: "These results suggest that actin modulation can suppress PLX4032 resistant cell viability, although resistant WM3248 cells did not show a clear synergistic effect due to their inherent sensitivity to cytochalasin D and blebbistatin." In addition, we have repeated the experiments presented in Fig. 7F using SKMEL28 cells. The new results from SKMEL28 also support our conclusion.

4. For TESK1 knock down (Figure 8), the WM3248 cell line shows only mild sensitivity, and not under all culturing condition tested. The inconsistency between cell lines, and the sensitivity of the results to the culture conditions, suggest that highly plastic melanoma cells could evolve adaptive resistant mechanisms to the strategy proposed. From this perspective, it is difficult to conclude that

TESK targeting is a promising approach. For the same reasons, it is overstatement to say that TESK1 knockdown exerted a profound effect.

We agree with this critical comment. We have rephrased “exerted a profound effect” to “suppressed”. In addition, we have included new statements on this issue in the discussion section: [“However, we also found an inconsistency in sensitivity to TESK1 inhibition between the two cell lines, and serum supplement influenced the effect of TESK1 knockdown.” “These results suggest that melanoma cells could evolve adaptive resistant mechanisms to TESK1 inhibition.”]

5. It cannot be claimed that YAP/TAZ knock down restores sensitivity to BRAF inhibition, as is done in the corresponding results sections bold subtitle, b/c this is not a consistent result across the two cell lines. It can be more reasonably claimed that resistant cells have higher dependency on YAP/TAZ. The results of Fig 4 overall suggest that resistant cells are more sensitive to YAP/TAZ knockdown, but do not support a true synergy/synthetic lethality with BRAF inhibition (see Fig 4D, individual and combined treatments with siYT and PLX are at best additive and mainly due to siYT). In regards to survival, what are the effects of siYT on the resistant cells (independent of PLX)? Fig 4A does not convey what is the effect on survival due to introduction of the siYT since the data in this panel is normalized.

We agree that YAP/TAZ dependency needs to be distinguished from synthetic lethality of YAP/TAZ depletion with BRAF inhibition. As shown in Figs. 4D and EV3F, YAP/TAZ inhibition alone suppressed resistant cell proliferation and viability without PLX4032 treatment. Thus, we have revised our manuscript to indicate that both resistant SKMEL28 and WM3248 cells have higher YAP/TAZ dependency than parental cells: “These results suggest that PLX4032 resistance is associated with higher YAP/TAZ dependency”. In regard to synthetic lethality, resistant SKMEL28 cells showed clear restoration of sensitivity to PLX4032 upon YAP/TAZ knockdown (Fig. 4A), however, as the Referee indicated, resistant WM3248 cells showed low levels of restoration. Therefore, we have revised our section title as “YAP/TAZ knockdown suppresses resistant cell viability, whereas constitutively active YAP induces PLX4032 resistance”. In addition, our final manuscript includes the following sentences: [“Dose-response analyses revealed that double-knockdown of YAP and TAZ, using two distinct siRNA sets, causes shifts in PLX4032 sensitivity in resistant cells (Figs. 4A and EV3A).” “A significant restoration of PLX4032 sensitivity after YAP/TAZ knockdown was noted in resistant SKMEL28 cells, and resistant WM3248 cells showed lower levels of restoration of PLX4032 sensitivity.”]

6. Experiments using other targeted therapies shown in Figures 4E,F, and G needs to be performed on parental cell lines as a control for specificity to the resistant lines. Experiments in Fig 4F and EV4C are missing the PLX plus Erlotinib control. The authors do not comment on the finding that neither EGFR nor AKT fully explain the BRAF inhibitor resistance since treatment with corresponding EGFR and/or AKT inhibitors only partially kills the cells. Most of the reduction in cell viability is through MK-2206 (AKTi) and the addition of the other drugs does not add much effect. As was done for MYC, qualifications of these results need to be added in the results and discussion sections: 'Our finding suggests that YAP may serve as an important regulator of EGFR and AKT pathway activity in BRAF inhibitor resistance models, but that additional pathways are likely also involved in mediating resistance to BRAF inhibition (Fig 4F).' 'MK-2206 **partially** suppressed resistant cell survival, and combination with Erlotinib and PLX4032 resulted in **only slightly** higher suppression of cell viability. **No treatment strongly suppressed cell viability**' We also depleted c-MYC expression by two independent c-MYC siRNAs (Fig EV4D), and observed a **slight** suppression of cell viability, but only in the **resistant SKMEL28 cells** (Fig 4G).'

We think our results in Figure 4E, F, and G are valid without parental cell controls, because these results demonstrate the alterations in molecular pathways upon YAP/TAZ knockdown in resistant cells. Because we have observed specific effect of YAP/TAZ knockdown on resistant cell viability and proliferation (Fig. 4A-D), we were aimed to analyze downstream pathway of YAP/TAZ knockdown in resistant cells, not in parental cells. Even if results presented in Figure 4E, F, and G are not specific for resistant cells and also observed in parental cells, our results would still have the same significance because they help us to understand YAP/TAZ-dependent molecular pathways in BRAF inhibitor resistance. According to the Referee's second comment, we have added PLX plus Erlotinib control to Figs. 4F and EV4C (we already had the data, but omitted them because the graph seemed too complex). We have also revised our manuscript as recommended: [“We treated resistant cells with EGFR inhibitor Erlotinib, and AKT inhibitor MK-2206 (Figs 4F and EV4C).” “MK-2206 partially suppressed resistant cell survival, and combined treatment with Erlotinib and

PLX4032 resulted in slightly higher suppression of cell viability.” “We also depleted c-MYC expression by two independent c-MYC siRNAs (Fig EV4D), and observed a reduction of cell viability in resistant SKMEL28 cells (Fig 4G).” “These results suggest that AKT, EGFR and c-MYC contribute to the survival of resistant cells, although inhibition of the pathways does not seem to be sufficient to overcome BRAF-inhibitor resistance.”]

7. Likewise, Figure 7 is difficult to adequately interpret due to a lack of parental controls. In contrast, Figure 8 does include parental controls. The authors state that only WM3248 shows sensitivity to Blebbistatin and CytoD, but in Fig 7G (as opposed to Fig 7E) SKMEL28 shows some sensitivity as well.

We understand Referee’s concern. Results from parental cells as a control may provide additional information on the biology of YAP/TAZ in melanoma cells. However, we think our conclusions in Fig. 7 are valid without parental cell control, because we aim to understand the nature of YAP/TAZ localization/activity/function in resistant cells in order to find new strategy for overcoming drug resistance. Moreover, our results (Fig. 4) clearly demonstrate that dependency on YAP/TAZ is greater in resistant cells than in parental cells. The difference in the experimental condition between Fig. 7E and 7G might explain why resistant SKMEL28 cells showed slightly different sensitivity to CytoD and Blebb in the absence of PLX4032. In Fig. 7G, cells were transfected with retroviral vector, which might sensitize cells from apoptotic pathways. In our final manuscript, we describe the discrepancy.

- Minor points

1. Results for Figure 3A-B need additional replicates and error bars, as done in the other panels in the figure.

According to the Referee’s suggestion, we have repeated the experiments and added error bars in the figure.

2. The authors do not comment on why increased serum levels alter the WM3248 parental and resistant lines differential response to siTESK1 knockdown, nor do they explain their reasoning behind testing higher serum levels.

Previous studies have shown that serum (FBS) potently upregulates YAP/TAZ activity (Yu et al., Cell. 2012, 150, 780-91) through GPCR signaling. In this study, SKMEL28 cells were grown in MEM supplemented with 10 % FBS, while WM3248 cells were grown in MCDB-153/L-15 supplemented with 2 % FBS. We reasoned that increasing serum concentration in WM3248 culture may alleviate cell viability loss after TESK1 knockdown, possibly by compensating downregulation of YAP/TAZ activity. As expected, the viability of both parental and resistant WM3248 cells increased in the presence of 10 % FBS after TESK1 knockdown. We have included our reasoning in the result section: “We reasoned that the inherent sensitivity of WM3248 cells to TESK1 knockdown might be alleviated by increasing the concentration of serum, which is known to promote YAP/TAZ activity (Yu et al, 2012).”

3. The GSEA results shown in Fig 3I are of borderline significance (NES = 1.4) and need to be acknowledged as such. Only one gene set is shown - the full GSEA results need to be shown in Table S3 for comparison, as done for the other enrichment analyses. The enrichment scores and statistical test values for 3I need to be stated in the legend.

We have included the full GSEA result in Table S3 for comparison. The GSEA identified only RB_DN and YAP signature gene sets as significantly enriched on resistant melanoma cells compared with parental cells. We also added the normalized enrichment score, nominal p-value and FDR q-value to the legend of Fig. 3I.

4. The statement in the abstract that "Knockdown of YAP/TAZ overcomes PLX4032 resistance" needs to be qualified since this would not be true of all versions of BRAF inhibitor resistance (e.g. NRAS mutation, BRAF alternative splicing). Also on the second line of page 7.

We have revised the sentences: [“Knockdown of YAP/TAZ suppressed the resistant melanoma cell viability” (in the abstract); and “To address the role of YAP/TAZ activation in BRAF inhibitor resistance, we next asked whether YAP/TAZ knockdown can suppress the viability of PLX4032 resistant melanoma cells.” (in the result)]

5. YAP/TAZ nuclear localization does not peak at 7 days for WM3248 as stated by the authors (Figure 3C and 3D).

We have revised the sentence: “Nuclear YAP/TAZ localization peaks after 7 days of PLX4032 treatment in SKMEL28 cells and after 14 days in WM3248 cells, which correspond to the progression of actin remodeling shown in Fig 2F.”

6. In Fig EV5C, TESK1 mRNA is several fold higher in WM3248 resistant cells than parental WM3248 and SKMEL28. Yet, the western blot in Fig EV5D does not show higher protein levels. This disconnect between mRNA and protein, combined with the inconsistent results seen between cell lines, indicate that a Western blot is needed to confirm reduction of TESK1 protein levels upon TESK1 knockdown.

We still do not understand the cause of the inconsistency between TESK1 mRNA and protein levels. To confirm that TESK1 siRNAs deplete TESK1 proteins, we performed a western blot analysis. As presented in our final manuscript (Fig. EV5A), TESK1 siRNAs indeed decreased TESK1 protein levels.

7. For Figure 8B, the authors used a z-score approach combined with an arbitrary threshold to filter for candidates. The rationale stated in their response to reviewer comments for not using a statistical test with multiple hypothesis correction was that “RNAi library screen data are not suitable for a statistical test followed by multiple hypothesis correction when the screen is done in duplicates.”

This statement is unclear. Consultation with a statistician will identify a statistical approach that can appropriately handle the replicate structure of the experiment.

Our screen data analysis was based on “Statistical methods for analysis of high-throughput RNA interference screens” (Nat Methods. 2009, 6(8): 569–575.). As we already mentioned, Z-score based analysis used in our study is a common approach for analyzing RNAi library screen experiments of lesser than triplicate. Major journals accept the analysis method. There are many recent examples: Cancer Cell 2015, 28, 129-40; Sci Signal 2012, 5, ra3; J Cell Biol 2013, 200, 505-522; EMBO 2014, 33, 181-197; Nat Cell Biol 2014, 16, 108-117.

8. There were no legends for the supplemental figures.

Our manuscript includes legends for the Supplemental Figures (Expanded View Figures) at the end of the main text.