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Discrete cytosolic macromolecular BRAF complexes exhibit distinct activities and composition

Britta Diedrich, Kristoffer T.G. Rigbolt, Michael Röring, Ricarda Herr, Stephanie Kaeser-Pebernard, Christine Gretzmeier, Robert F Murphy, Tilman Brummer, Jörn Dengjel

Corresponding author: Jörn Dengjel, University of Fribourg & Tilman Brummer, Albert-Ludwigs-University, Freiburg

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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 13 June 2016

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 while the referees appreciate the analysis they also bring up important concerns that need to be resolved in order to consider publication here. One issue raised is that the dataset relies to a large degree on overexpression analysis and that further support using endogenous protein levels is needed to support the main conclusions. There are also a number of technical concerns raised. Should you be able to address the concerns raised in full then I 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 concerns at this stage.

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:

In this paper the authors characterise and compare protein complexes between cells overexpressing BRAF or BRAF V600E. Using SEC-PCP-SILAC they find that BRAF containing complexes are the most different between the two conditions, and further analyse BRAF and BRAF V600E interacting proteins by co-IP MS and biochemical assays, such as Blue-Native gels, Western blotting and kinase assays. They then further analyse the effects of RAF and HSP90 inhibitor drugs on the BRAF complexes and their catalytic activities. This is an interesting and very timely paper given the huge efforts to understand BRAF regulation. However, it contains several inconsistencies and over-interpretation of the data that preclude its publication in the current form.

Specific comments

Fig. 1C. Quantification of 1945 proteins may not be sufficient to claim that BRAF overexpression does not change global protein expression. At this depth of analysis most of these proteins will be abundant protein species, whose concentrations may not change significantly in response to perturbations. A more informative comparison would be to assess the expression changes of BRAF interacting proteins.

Fig. 1C. A ca. 9 fold overexpression of BRAF proteins may lead to unphysiological effects. This concern is supported by the kinase activity of wt BRAF being similar to the activity of V600E suggesting a loss of regulation due to overexpression. The authors need to assess the influence of this high BRAF overexpression, e.g. by examining BRAF protein interactions and carefully validate the results obtained with overexpression against endogenous co-IPs.

Fig. 2B. What do the profiles exactly show? Presumably, each line represents a ribosomal protein? Which non-ribosomal proteins were detected in these fractions sharing similar elution profiles?

Fig. 2C. What are the normalised ratios? Ratios between what? Normalised to what?

Fig. 3. This figure also raises concerns about the effects of BRAF overexpression and the limited sensitivity of the MS experiments. While the binding of 14-3-3 and Cdc37 to BRAF wt and V600E is clearly different in co-IP Western blot experiments, it is far from being exclusive to the low and high MW BRAF complexes as suggested by the MS experiment. In addition, binding of 14-3-3 and Cdc37 to both BRAF and BRAF V600E has been shown previously in the literature, e.g. {Brummer, 2006 #2;Polier, 2013 #1}. The BRAF interaction network obtained by co-IP MS shown in Fig. 4B contains several 14-3-3 proteins interacting equally with both BRAF wt and V600E. Thus, these results may suggest that the sensitivity of the MS experiment is fairly limited and/or that the high overexpression of BRAF may affect some interactions.

Fig. 4. The BRAF interacting networks obtained by co-IP MS and SEC-PCP SILAC should be compared. This will be a good indicator of the sensitivity and specificity of the methods.

Fig. 4E. According to the Western blot of the SEC fractions BRAF wt and V600E are equally distributed between the low and high MW complexes. This is in contrast to the results shown in Fig. 3 and the conclusion on p12: "Taken together, BRAF localizes to structurally discrete macromolecular protein complexes. A considerable part of oncogenic BRAFV600E resides in a complex of approx. 600 kDa together with CDC37 and HSP90. In contrast, the majority of BRAFWT is found in a complex of approx. 200 kDa together with 14-3-3 proteins and MEK."

Fig. 5A. What is the right part of the panel labelled DMSO? This is not explained anywhere.

Fig. 6H. The enhancement of pMEK in lane 2 does not change pERK. Why?

Referee #2:

This manuscript describes the application of PCP-SILAC to separate functions and activities of BRAF. It is really an elegant study that shows the power of an unbiased approach combined with functional hypothesis testing. My expertise is more with the proteomics so I will focus on that. The whole PCP-SILAC approach is very well done though.

Comments
1. The introduction seems a little bit long and maybe could be cut 20%?

2. This manuscript really hinges on the differential behaviour of BRAF in the WT vs. V600E conditions. Is it possible that a portion of the different size exclusion chromatograms of BRAF can be due to the peptide containing V600(E)? It seems unlikely since there appears to be 52 peptides attributed to BRAF in Table S1 but this should be confirmed.

3. The raw MS data should be uploaded to ProteomeXchange prior to acceptance

Referee #3:

Diedrich and colleagues analyze discrete cytosolic complexes of either BRAF WT or the oncogenic mutant V600E. Using a newly developed mass spectrometry technique (SEC-PCP-SILAC) they were clearly able to show two discrete soluble pools of BRAF and a differential distribution of either WT of V600E into these pools. In the higher MW pool, BRAF V600E is shown to interact with HSP90/CDC37 consistent with previous literature showing that BRAF V600E has increased dependency (over WT) on the HSP folding machinery. The lower MW pool also shows discrete composition, with 14-3-3 proteins and the known 14-3-3 interacting phosphorylation marks on BRAF exclusively in this lower MW pool. Inhibitors targeting specific components of these pools altered the relative abundance of BRAF in these specific fractions. Both the HSP90 inhibitor 17-AAG and BRAF inhibitor decreased the abundance of BRAF V600E in the higher MW pool. In contrast, Trametinib increased the abundance of both BRAF WT and V600E in the higher MW pool. Interestingly, while BRAF WT resides mostly in the low MW pool, induction of ectopically expressed KRAS G12V (or basally in the NRAS Q61K mutant cell line SBcl2) shifts BRAF WT into a high MW pool (albeit it wasn't directly shown that the composition of this pool is similar to the high MW pool of BRAF V600E).

Overall, this paper makes an interesting observation that BRAF either WT or V600E can reside in discrete cytoplasmic pools (with unique binding partners and post-translational modifications) and that these pools undergo dynamic re-arrangements upon either inhibitor treatment or pathway activation (in the case of WT). These observations are interesting and expand upon previously published work.

My two major concerns are that the bulk of the experiments including the characterization of the high and low molecular weight complexes are conducted using overexpression of BRAF or BRAF-V600E which may result in findings that are not relevant to endogenous expression levels. Secondly, some of the gel quantitation in its current form lacks the robustness and needs additional measurements to merit drawing specific conclusions from these experiments (more details below).

Specific comments that need to be addressed are highlighted below:

• Figure 1A and 1B, the difference between WT and V600E pMEK or pERK is very subtle which is surprising. Overexpression of V600E has been shown by others to dramatically increase pMEK levels over WT. Is there an explanation for this low levels of activation?
• Figure 3H: the native gels here show BRAF-V600E migrating primarily as a low molecular weight species in contrast to previous observations. Further the expression level of the BRAF-V600E/S365A is lower compared to BRAF-V600E (is this mutant more unstable?) and therefore difficult to conclude that the mutation alone impacts its ability to migrate in the low or high MW fractions.
• Figure 4E: In the vector alone there is a significant amount of CDC37 and FKBP5 in the HWM fractions and a significant amount of PPP2R2A and 14-3-3 in the LMW fractions (nearly identical to BRAF WT or V600E). I suppose a simple explanation is that a small portion is coming from the low level of endogenous BRAF. However, from this it is hard to argue that these proteins are co-eluting

due to the presence of the ectopically expressed BRAF. I think it is important to show not just the quantification of enrichment in 4F, but also a side-by-side analysis of all 6 fractions in 4E using anti-HA affinity matrix (what is background binding in the vector alone control fractions to the HA beads?).
• In Figure 4F drastically different behaviors for MEK1 (MAP2K1) and MEK2 (MAPK2K2) are observed. This should be addressed and doesn't fit with the text (page 11) where it states V600E weakens interaction between BRAF-V600E and MEK (as has also already been shown by others).
• Also, with Figure 4F only quantifications of IPs from HA-V600E from low and high MW fractions are shown. Given that many of the conclusions are about differences between WT and V600E it would be nice to see the relative abundance of various proteins from IPs of HA-WT as well.
• In Figure 5A, the amount of BRAF-WT vs. BRAF-V600E IP'd from cells is quite different and all quantitation (CDC37, 14-3-3) needs to be normalized to the amount of BRAF immunoprecipitated. Further, Trametinib increases the amount of both Wt and V600E bound to Cdc37. However, the quantification (Figure 5B) doesn't show nearly the same degree of enrichment or show that it is significant. This is an interesting finding and it should be clarified.
• Figure 5E-It appears that total MEK levels are down in all the treatment conditions. The pMEK levels should be normalized to total MEK levels for quantitation.
• In Figures 6A & B the differences are subtle and I find it hard to argue there is much of a difference in activity between V600E in the high and low MW fractions. Further it is unclear what "AU" means in the quantitation. To measure kinase activity, one would need to quantitate pMEK and normalize to enzyme concentration. Is there linearity in the enzyme reaction? Is the reaction run at ATP Km? A 1.7-fold difference in activity seems within experimental error in this case.
• In Figure 6C, is the composition of the high MW fractions of BRAF WT induced by KRAS G12V similar to the high MW fractions basally for BRAF V600E? The way it is written it leads the reader to assume this, but it should be tested by IP from the high MW fraction (similar to the IPs in Figure 4F).
• Figure 6G: The data here from the native gels is not convincing at all. What is the purpose of the EtOH condition? This needs to be clarified.
• As a general statement, I appreciate how the use of the isogenic lines allows a direct comparison of changes caused just by the ectopically expressed protein, but I am concerned about how overexpression could cause a shift into different MW fractions. While the authors evaluated changes in global protein abundance upon BRAF overexpression, what has not been addressed is how the stoichiometry of complexes may be altered upon overexpression of BRAF or BRAF-V600E (especially with 9-fold higher levels of BRAF). In particular, we already know that the V600E mutation induces a different conformation of BRAF that increases the dependency on folding machinery (CDC37/HSP90). I imagine overexpression is only going to enhance this dependency. The validation of some of the drug observations in the SBcl2 cell line and MEFs is nice, but I feel it would be beneficial to see comparison of cells lines with "normal" BRAF WT or V600E expression levels to the current overexpression system earlier in the paper (Figure 2-4).

1st Revision - authors' response 24 October 2016

Referee #1:

In this paper the authors characterise and compare protein complexes between cells overexpressing BRAF or BRAF V600E. Using SEC-PCP-SILAC they find that BRAF containing complexes are the most different between the two conditions, and further analyse BRAF and BRAF V600E interacting proteins by co-IP MS and biochemical assays, such as Blue-Native gels, Western blotting and kinase assays. They then further analyse the effects of RAF and HSP90 inhibitor drugs on the BRAF complexes and their catalytic activities. This is an interesting and very timely paper given the huge efforts to understand BRAF regulation. However, it contains several inconsistencies and over-interpretation of the data that preclude its publication in the current form.

We would like to thank referee #1 for the interest in our manuscript and the helpful comments to which we respond point-by-point below (in blue).

Specific comments

Fig. 1C. Quantification of 1945 proteins may not be sufficient to claim that BRAF overexpression does not change global protein expression. At this depth of analysis most of these proteins will be

abundant protein species, whose concentrations may not change significantly in response to perturbations. A more informative comparison would be to assess the expression changes of BRAF interacting proteins.

We would like to thank referee #1 for this important comment and agree with the reviewer that the dataset is limited. As we had the same concern, we specifically checked potential abundance differences of known BRAF binding partners and included this as Figure 1D in the revised manuscript. We generated an enlarged **new Figure 1D** now also displaying amongst others the seven 14-3-3 proteins, CDC37, and HSP90. We do not detect significant abundance differences of BRAF binding partners in cells either overexpressing $BRAF^{WT}$ or $BRAF^{V600E}$. Thus, we conclude that BRAF overexpression does not change the abundance of BRAF interactome members, in particular those of central relevance to this manuscript.

Fig. 1C. A ca. 9 fold overexpression of BRAF proteins may lead to unphysiological effects. This concern is supported by the kinase activity of wt BRAF being similar to the activity of V600E suggesting a loss of regulation due to overexpression. The authors need to assess the influence of this high BRAF overexpression, e.g. by examining BRAF protein interactions and carefully validate the results obtained with overexpression against endogenous co-IPs.

We would like to thank referee #1 for this comment and fully agree that a 9-fold overexpression might lead to non-physiological effects. Therefore, we had established a murine embryonic fibroblast system allowing us to study cells either expressing endogenous $BRAF^{WT}$ or $BRAF^{V600E}$ in an isogenic system. In the revised manuscript, we have extensively used this *Braf*^{floxV600E}; *Rosa*26::CreERT2 MEF system to study complexes containing endogenous BRAF proteins (please see the **new Figure 3** for further details). Importantly, our new results obtained in the MEFs are in agreement with the CaCo-2-tet overexpression system, indicating that we were not investigating overexpression artefacts in the CaCo-2-tet system.

Like referee #1, we were initially also surprised about the small difference between the MEK and ERK phosphorylation between $BRAF^{WT}$ or $BRAF^{V600E}$ expressing CaCo-2-tet cells. However, we would like to point out that there is a drastic differential in MEK and ERK phosphorylation levels in $BRAF^{WT}$ or $BRAF^{V600E}$ expressing cells at 12 h (**Figure 1A/B**). This clearly shows that oncogenic $BRAF^{V600E}$ is more potent than its wildtype counterpart in CaCo-2tet cells. Nevertheless, we agree that this differential is reduced with increasing culture time. As several feedback and robustness phenomena have been identified in the ERK pathway in recent years (Fey et al., 2016, Fritsche-Guenther et al., 2011), there might be several reasons as to why this might be the case. Firstly, the strong initial pERK signal in the $BRAF^{V600E}$ expressing cells might induce dual specificity phosphatases to a larger extent than in $BRAF^{WT}$ expressing cells, reducing the differential in pERK levels. Furthermore, the effects on pMEK levels could be explained by the negative feedback from ERK to MEK1 (Catalanotti et al., 2009) , which we would expect to be more relevant in $BRAF^{V600E}$ expressing cells. At the editor's discretion, we could add these aspects to the discussion part of the manuscript.

Lastly, we would like to point out that a 9-fold overexpression might be non-physiological but still pathophysiological relevant as overexpression of $BRAF^{WT}$ has been observed in various tumours and, importantly, as the expression of $BRAF^{V600E}$ can be increased by more than 10-fold during tumour progression, for example as result to drug adaptation (Little et al., 2011, Moriceau et al., 2015). We have mentioned these papers on p.8 of the revised manuscript.

Fig. 2B. What do the profiles exactly show? Presumably, each line represents a ribosomal protein? Which non-ribosomal proteins were detected in these fractions sharing similar elution profiles?

We would like to thank referee #1 for this comment and apologise for not providing enough details. Indeed, each line represents a ribosomal protein. All proteins that were also detected in the respective fractions can be found in Supplemental Table 2 in which we give details about protein complexes defined by co-elution, e.g. complex 1 containing the large ribosomal subunit contains 26 proteins of the 60S ribosomal subunit and 28 associated proteins, complex 4 containing the small ribosomal subunit contains 21 proteins of the 40S ribosomal subunit and 6 associated proteins

We modified the figure legend as follows:

"SEC-PCP-SILAC elution profiles of 60S (green, 26 proteins) and 40S (blue, 21 proteins) ribosomal subunits. Each line represents the elution profile of a specific protein (see supplemental tables 1 and 2 for complete lists)."

Fig. 2C. What are the normalised ratios? Ratios between what? Normalised to what?

We modified the figure legend giving more details:
"As an example WT elution data from replicate 1 is shown. SILAC protein ratios of WT proteins versus the internal standard were normalized to one, highlighting the relative abundance of a specific protein in all SEC fractions."

Fig. 3. This figure also raises concerns about the effects of BRAF overexpression and the limited sensitivity of the MS experiments. While the binding of 14-3-3 and Cdc37 to BRAF wt and V600E is clearly different in co-IP Western blot experiments, it is far from being exclusive to the low and high MW BRAF complexes as suggested by the MS experiment. In addition, binding of 14-3-3 and Cdc37 to both BRAF and BRAF V600E has been shown previously in the literature, e.g. {Brummer, 2006 #2;Polier, 2013 #1}. The BRAF interaction network obtained by co-IP MS shown in Fig. 4B contains several 14-3-3 proteins interacting equally with both BRAF wt and V600E. Thus, these results may suggest that the sensitivity of the MS experiment is fairly limited and/or that the high overexpression of BRAF may affect some interactions.

We would like to thank referee #1 for this comment and agree that our initial statement of exclusivity of 14-3-3 proteins was indeed an overstatement. We do detect differential distribution and binding, as also shown in the **new Figure 3F-J**, but we also detect some 14-3-3 proteins in the large complex, although far less abundant. We have rewritten this section in the manuscript (**p. 11 first line; page 13 and 19 and 20 first paragraphs**) accordingly. CDC37 and HSP90, however, appear to exclusively localize to the large complex.

Concerning the original Figure 3F, this may have been a misunderstanding. Here we performed CO-IPs from whole cell lysates and not from SEC fractions. So we do expect to detect 14-3-3 and CDC37 in both, WT and V600E complexes. We clarified this in the figure legend:
"14-3-3 proteins interact stronger with WT, CDC37 stronger with V600E as shown by anti-HA and anti-CDC37 IPs from whole cell lysate."

Finally, we would like to point out that we never intended that the data of our manuscript should be understood as a contradiction to previous findings, incl. our own, but rather as a significant refinement of current models of the BRAF activation cycle. We fully agree with referee #1 that binding of 14-3-3 and Cdc37 to both $BRAF^{WT}$ and $BRAF^{V600E}$ has been shown previously in the literature as we have also stated in the original and revised manuscript with several citations, incl. Brummer et al., 2006 and Polier et al. 2013. As co-immunoprecipitation and GST-14-3-3 pulldown experiments from whole cell lysate cannot discriminate between the HMW and LMW complexes of both $BRAF^{WT}$ and $BRAF^{V600E}$, HSP90/CDC37 and 14-3-3 will be always co-purified. With SEC-PCP-SILAC, however, we are now able to discern these distinct complexes and to subject them, in contrast to BN-PAGE, to a largely unbiased analysis for their composition.

Fig. 4. The BRAF interacting networks obtained by co-IP MS and SEC-PCP SILAC should be compared. This will be a good indicator of the sensitivity and specificity of the methods.

We would like to thank referee #1 for this important suggestion. By comparing SEC and co-IP data we identified the differential distribution of FKBP5 and PPP2R2A (Figure 4B-D and **new Figure 4E-F**). We also performed co-IPs from SEC fractions and included a **new Figure 4H**. **New Figures 4G and 4H** highlight that 14-3-3 binding to the large and small complex is differential for both V600E and WT complexes. However, it is not exclusive as correctly suggested by the reviewer. Again, CDC37 and HSP90 appear to be exclusively in the large complex. We have also modified the legend of **Figure 4** to allow a better understanding of the experimental procedures.

Fig. 4E. According to the Western blot of the SEC fractions BRAF wt and V600E are equally distributed between the low and high MW complexes. This is in contrast to the results shown in Fig. 3 and the conclusion on p12: "Taken together, BRAF localizes to structurally discrete macromolecular protein complexes. A considerable part of oncogenic BRAFV600E resides in a

complex of approx. 600 kDa together with CDC37 and HSP90. In contrast, the majority of BRAFWT is found in a complex of approx. 200 kDa together with 14-3-3 proteins and MEK."

We would like to thank referee #1 for alerting us to the fact that we have not provided sufficient details to understand the data in Figure 4E. First of all, the differential distribution of WT and V600E is nicely illustrated in the new **Figure 3B** for CaCo-2-tet cells using ectopic expression and in the **new Figure 3F** for MEFs using endogenous expression for both $BRAF^{WT}$ and $BRAF^{V600E}$. Thus, the differential distribution patterns of wildtype and oncogenic BRAF originally discovered in ectopic expression experiments could be reproduced for endogenous BRAF. However, we fully agree with referee #1 that the original Figure 4E raises another impression. In the SEC-PCP-SILAC experiments, we analysed single fractions generating detailed profiles. For the blot shown in the old Figure 4E (**new Figure 4I**), however, we combined several fractions to increase protein amount for western blotting and also normalized each loaded sample to protein amount. While this western blot analysis nicely shows the differential distribution of binding partners, this "bulk" approach might lead to a misinterpretation of BRAF distribution. We have now provided this information in the revised Figure legend.

Fig. 5A. What is the right part of the panel labelled DMSO? This is not explained anywhere.

Inhibitors were dissolved in DMSO and final DMSO concentration was 0.01%. Hence, DMSO treatment served as vehicle control. We rewrote the figure legend as follows:
"DMSO treatment served as vehicle control."
Also, we added respective information to Materials and Methods section.

Fig. 6H. The enhancement of pMEK in lane 2 does not change pERK. Why?

We would like to thank referee #1 for this comment and point out that there is a growing notion in the field that pMEK represents a better direct readout for RAF activity than pERK. pERK levels are well-buffered by various mechanisms such as immediate early and delayed feedback loops involving various phosphatases such as dual specificity phosphatases (Birtwistle & Kolch, 2011) (Schilling et al., 2009). The phenomenon that pMEK and pERK levels do not always correlate has been particularly observed in $BRAF^{V600E}$ expressing human and murine cells (Hernandez et al., 2016, Herr et al., 2015, Pratilas et al., 2009). In addition, we would like to point out that these immortalised MEFs were not subject to serum starvation, but were grown in conventional culture medium containing foetal calf serum. This potentially blurs effects at the pERK level by the aforementioned mechanisms. If desired, we could supply a comment in the manuscript.

Referee #2:

This manuscript describes the application of PCP-SILAC to separate functions and activities of BRAF. It is really an elegant study that shows the power of an unbiased approach combined with functional hypothesis testing. My expertise is more with the proteomics so I will focus on that. The whole PCP-SILAC approach is very well done though.

We would like to thank referee #2 for the interest in our manuscript, for appreciating the power of our approach and the helpful comments to which we respond point-by-point below (in blue).

1. The introduction seems a little bit long and maybe could be cut 20%?

We would like to thank referee #2 for this comment and have cut the introduction by 30%.

2. This manuscript really hinges on the differential behaviour of BRAF in the WT vs. V600E conditions. Is it possible that a portion of the different size exclusion chromatograms of BRAF can be due to the peptide containing V600(E)? It seems unlikely since there appears to be 52 peptides attributed to BRAF in Table S1 but this should be confirmed.

We would like to thank referee #2 for this comment. We only identified the peptide containing valine 600, which behaved like the other BRAF peptides in WT samples. As we did not identify the glutamate 600 peptide data concerning the HMW complex cannot be influenced by the mutant version.

3. The raw MS data should be uploaded to ProteomeXchange prior to acceptance

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD004585. We added a respective statement to the text. Currently, the data is private and can be accessed by:

Project Name: BRAF complexes
Project accession: PXD004585
Reviewer account details:
Username: reviewer86913@ebi.ac.uk
Password: EuLqOwWP

Upon acceptance of the paper, we will make the data publically available.

Referee #3:

Diedrich and colleagues analyze discrete cytosolic complexes of either BRAF WT or the oncogenic mutant V600E. Using a newly developed mass spectrometry technique (SEC-PCP-SILAC) they were clearly able to show two discrete soluble pools of BRAF and a differential distribution of either WT of V600E into these pools. In the higher MW pool, BRAF V600E is shown to interact with HSP90/CDC37 consistent with previous literature showing that BRAF V600E has increased dependency (over WT) on the HSP folding machinery. The lower MW pool also shows discrete composition, with 14-3-3 proteins and the known 14-3-3 interacting phosphorylation marks on BRAF exclusively in this lower MW pool. Inhibitors targeting specific components of these pools altered the relative abundance of BRAF in these specific fractions. Both the HSP90 inhibitor 17-AAG and BRAF inhibitor decreased the abundance of BRAF V600E in the higher MW pool. In contrast, Trametinib increased the abundance of both BRAF WT and V600E in the higher MW pool. Interestingly, while BRAF WT resides mostly in the low MW pool, induction of ectopically expressed KRAS G12V (or basally in the NRAS Q61K mutant cell line SBcl2) shifts BRAF WT into a high MW pool (albeit it wasn't directly shown that the composition of this pool is similar to the high MW pool of BRAF V600E).

Overall, this paper makes an interesting observation that BRAF either WT or V600E can reside in discrete cytoplasmic pools (with unique binding partners and post-translational modifications) and that these pools undergo dynamic re-arrangements upon either inhibitor treatment or pathway activation (in the case of WT). These observations are interesting and expand upon previously published work.

My two major concerns are that the bulk of the experiments including the characterization of the high and low molecular weight complexes are conducted using overexpression of BRAF or BRAF-V600E which may result in findings that are not relevant to endogenous expression levels. Secondly, some of the gel quantitation in its current form lacks the robustness and needs additional measurements to merit drawing specific conclusions from these experiments (more details below).
Specific comments that need to be addressed are highlighted below:

We would like to thank referee #3 for the interest in our manuscript and the helpful comments to which we respond point-by-point below (in blue). In essence, we have now included several data sets investigating complexes formed by endogenous BRAF proteins and provide additional measurements and quantitation.

Figure 1A and 1B, the difference between WT and V600E pMEK or pERK is very subtle which is surprising. Overexpression of V600E has been shown by others to dramatically increase pMEK levels over WT. Is there an explanation for this low levels of activation?

We would like to thank referee #3 for this comment, which was in a similar way also raised by referee #1. Given our experiences with other cell lines ectopically expressing oncogenic BRAF, we were also surprised about the small differential between the MEK and ERK phosphorylation between $BRAF^{WT}$ or $BRAF^{V600E}$ expressing CaCo-2-tet cells. However, we would like to point out that there is a drastic differential between in MEK and ERK phosphorylation levels in $BRAF^{WT}$ or $BRAF^{V600E}$ expressing cells at 12 h (**Figure 1A/B**). This demonstrates that oncogenic $BRAF^{V600E}$ is more active than its wildtype counterpart in CaCo-2tet cells. Why this differential is reduced with increasing culture time remains an open question for future studies and could be explained by the several feedback and

robustness phenomena in the ERK pathway (Fey et al., 2016, Fritsche-Guenther et al., 2011). At the editor's discretion (because of the already high word count), we could discuss these aspects further in the manuscript.

Figure 3H: the native gels here show BRAF-V600E migrating primarily as a low molecular weight species in contrast to previous observations. Further the expression level of the BRAF-V600E/S365A is lower compared to BRAF-V600E (is this mutant more unstable?) and therefore difficult to conclude that the mutation alone impacts its ability to migrate in the low or high MW fractions.

We always detect a clear high molecular weight complex in V600E cells, which is significantly reduced in WT cells. This is true for cells expressing endogenous or ectopic BRAF proteins (see **new Figure 3F**). Due to transient expression in Plat-E cells, relative complex distribution may vary. We repeated the experiment identifying again a pronounced large complex in $BRAF^{V600E}$ and $BRAF^{S365A}$ expressing cells (see **new Figure 3H**). Loading controls using SDS-PAGE indicate similar abundance of BRAF variants, also of $BRAF^{S365A}$ and $BRAF^{V600E/S729A}$. The BRAF-V600E/S365A was neither tested in the original submission nor in the revised manuscript. We assume that referee #3 was referring to $BRAF^{V600E/S729A}$, which is indeed slightly less expressed than $BRAF^{V600E}$. However, we have no data supporting that this mutant is less stable.

Figure 4E: In the vector alone there is a significant amount of CDC37 and FKBP5 in the HWM fractions and a significant amount of PPP2R2A and 14-3-3 in the LMW fractions (nearly identical to BRAF WT or V600E). I suppose a simple explanation is that a small portion is coming from the low level of endogenous BRAF. However, from this it is hard to argue that these proteins are co-eluting due to the presence of the ectopically expressed BRAF. I think it is important to show not just the quantification of enrichment in 4F, but also a side-by-side analysis of all 6 fractions in 4E using anti-HA affinity matrix (what is background binding in the vector alone control fractions to the HA beads?).

Figure 4E (now **new Figure 4I**) was a bulk analysis of pooled SEC fractions which might have distorted the view. We included a detailed comparison of SEC fractions of endogenous BRAF variants and binding partners in **new Figure 3**. Also, as suggested by the reviewer, we performed anti-HA IPs from low and high molecular weight fractions and quantified interacting proteins. These experiments are include as Figure 4G for V600E cells and as **new Figure 4H** for WT cells.

WT and V600E complexes behave similar, the differential distribution of proteins being more clear in V600E complexes. We have rephrased this section of the manuscript, clearly stating that 14-3-3 proteins localize differentially but NOT exclusively to the large and small complexes. CDC37-HSP90 appears to localize almost exclusively to the large complexes.

In Figure 4F drastically different behaviors for MEK1 (MAP2K1) and MEK2 (MAPK2K2) are observed. This should be addressed and doesn't fit with the text (page 11) where it states V600E weakens interaction between BRAF-V600E and MEK (as has also already been shown by others).

In Figure 4F (now **new Figure 4G**) we only show V600E complexes. We did include now also the analysis of WT complexes (please see our statement to the previous comment by referee #3).

Figure 4B shows the differential distribution of MEK1 and 2 between WT and V600E. We elaborated on this in more detail in the results section (now on p. 12) and by referring to Haling et al. (2014), showing that increased MEK phosphorylation weakens the BRAF-MEK interaction. However, we fully agree that this does not explain the contrasting abundances of MEK1 and MEK2 derived peptides. We show now that MEK1 derived peptides were detected only in the LMW complex of $BRAF^{WT}$ but not in $BRAF^{V600E}$, while MEK2 derived peptides were detected in the LMW of both. This is an interesting observation in the light of the contrasting roles of both MEK isoforms in murine development and the preference of RAF kinases for MEK1 in ERK pathway signalling and oncogenic transformation (Belanger et al., 2003, Catalanotti et al., 2009, Caunt et al., 2015, Jelinek et al., 1994, Wu et al., 1996). In the context of active RAF complexes, however, this presumably higher affinity of MEK1 to RAF could be reduced in a "kiss-and-run" scenario in which MEK1 is rapidly recruited as a substrate and readily released as a phosphorylated product from the BRAF complex. A recent paper showing that the presence

of acetoacetate promotes the affinity of MEK1 to $BRAF^{V600E}$ further illustrates that this interaction is more dynamically regulated than originally anticipated (Kang et al., 2015). Thus, the absence of MEK1 derived peptides could simply reflect a higher turnover of MEK1 in the active BRAF complex. Our data, however, do not exclude the presence of MEK1 in the HMW of both proteins or the LMW of $BRAF^{V600E}$, but rather suggest that these peptides, potentially due to a more transient interaction of both kinases in the HMW, fall below the significance threshold. This needs to be addressed in the future studies, e.g. by establishing protocols combining crosslinkers with SEC-PCP-SILAC. We could include these aspects in the discussion, however, in order to keep the manuscript as concise as possible and because we feel that more studies are needed to corroborate this model, we would prefer to leave that aspect out of the current manuscript.

Also, with Figure 4F only quantifications of IPs from HA-V600E from low and high MW fractions are shown. Given that many of the conclusions are about differences between WT and V600E it would be nice to see the relative abundance of various proteins from IPs of HA-WT as well.

Please see statements above. As suggested we included a **new Figure 4H** showing the relative abundance of various proteins in $BRAF^{WT}$ complexes.

In Figure 5A, the amount of BRAF-WT vs. BRAF-V600E IP'd from cells is quite different and all quantitation (CDC37, 14-3-3) needs to be normalized to the amount of BRAF immunoprecipitated. Further, Trametinib increases the amount of both Wt and V600E bound to Cdc37. However, the quantification (Figure 5B) doesn't show nearly the same degree of enrichment or show that it is significant. This is an interesting finding and it should be clarified.

We would like to thank referee #3 for this comment. Indeed, we did normalize detected protein abundances to respective BRAF levels (Figure 5B). Upon closer re-inspection of the experiments we realised that the blot shown for CDC37 in the original submission was, in terms of the differential binding of Cdc37 to BRAF, not very representative for all three performed experiments. Hence the quantification shown in Figure 5B differed from the presented blot and this explains also the rather larger error bar in the quantification. We have now exchanged the CDC37 blot to show a more representative version. We would like to emphasize that the effect of trametinib was seen in all three independent experiments, albeit to a different degree.

Figure 5E-It appears that total MEK levels are down in all the treatment conditions. The pMEK levels should be normalized to total MEK levels for quantitation.

We would like to thank referee #3 for this comment as this western blot result also is not in line with our mass spectrometry experiments showing that MEK levels stay constant over the timeframe of the experiments. We have recently realized that the antibody used to detect MEK does not recognize MEK in cells with high BRAF activity. Unfortunately, the epitope or immunogen sequence for this widely used MEK1 antibody is not available, but given the high number of MEK phosphorylation sites (see www.phosphosite.org for details), we suspected that the stronger MEK becomes phosphorylated the weaker the MEK band appears. Indeed, we could confirm this hypothesis by treating our samples with λ-phosphatase, showing that dephosphorylation restores the original sensitivity of the antibody (**new Figure S1**). Under these circumstances, we think that it would not be adequate to normalise pMEK on total MEK levels. We also comment on this on p.8 of the revised manuscript as we think that this finding represents an important information for the field and demonstrates how MS based approaches can improve the interpretation of antibody based techniques.

In Figures 6A & B the differences are subtle and I find it hard to argue there is much of a difference in activity between V600E in the high and low MW fractions. Further it is unclear what "AU" means in the quantitation. To measure kinase activity, one would need to quantitate pMEK and normalize to enzyme concentration. Is there linearity in the enzyme reaction? Is the reaction run at ATP Km? A 1.7-fold difference in activity seems within experimental error in this case.

We would like to thank referee #3 for this comment. AU means arbitrary units. As indicated by the quantification the detected differences are statistical significant. The reactions were run at the near-physiological ATP concentration of 2.5 mM, which is two to three log-units over the reported micromolar K_m for $BRAF^{WT}$ and $BRAF^{V600E}$. Consequently, our chosen ATP concentration cannot be considered as kinase reaction

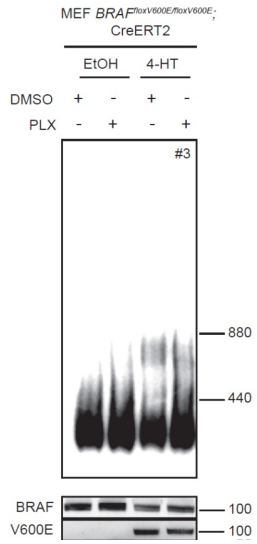
limiting. In addition we included a **new supplemental Figure S6** performing an *in vitro* kinase assay plus the addition of vemurafenib to highlight that we indeed measure BRAF activity.
However, we completely agree that a 1.7-fold change does not appear too dramatic. However, one has to keep in mind that we compare different versions of $BRAF^{V600E}$, a hyperactive kinase. Thus, one could imagine that it is difficult to boost kinase activity even further. Moreover, the differential for $BRAF^{WT}$ in the HMW and LMW of complexes purified from $KRAS^{G12V}$ expressing cells is already twofold, which is a relatively high differential for wildtype BRAF, given that BRAF *in vitro* kinase assays show already a high level of activity of the wildtype protein in the basal state, which not always reflects its biological activity (Mercer & Pritchard, 2003). Given that more research, probably including genetic approaches affecting the ratio between HMW and LMW complexes, will be required to address the functional significance of the differences in kinase activity, we weakened our statements (**Abstract page 3**) concerning kinase activity differences to better take the small differences into consideration.

In Figure 6C, is the composition of the high MW fractions of BRAF WT induced by KRAS G12V similar to the high MW fractions basally for BRAF V600E? The way it is written it leads the reader to assume this, but it should be tested by IP from the high MW fraction (similar to the IPs in Figure 4F).

This is indeed a very interesting experiment. However, as the manuscript already presents extensive datasets, we would like to address this in detail in a follow-up publication. Hence, we decided to weaken our statements and explicitly discuss that we cannot rule out that $KRAS^{G12V}$ induced WT complexes might differ in their composition from $BRAF^{V600E}$ complexes. Therefore, we added the following statement:
"However, as we did not perform detailed interactome studies of the different complexes in all cell lines/drugs tested we cannot rule out that the similar sized complexes differ in single components. This has to be addressed by future studies."

Figure 6G: The data here from the native gels is not convincing at all. What is the purpose of the EtOH condition? This needs to be clarified.

EtOH was used as vehicle control as 4-HT was dissolved in EtOH. We added a respective statement to the figure legend. We can reproducibly show the differential complexes upon endogenous V600E expression (see Figure below). However, one can argue about the quality of BN-PAGE analyses:



To better show the differential distribution of endogenous BRAF variants, we repeated SEC-PCP-SILAC analyses using the MEF system (see **new Figure 3F-I**). Please see also response to comment below.

As a general statement, I appreciate how the use of the isogenic lines allows a direct comparison of changes caused just by the ectopically expressed protein, but I am concerned about how overexpression could cause a shift into different MW fractions. While the authors evaluated changes in global protein abundance upon BRAF overexpression, what has not been addressed is how the

stoichiometry of complexes may be altered upon overexpression of BRAF or BRAF-V600E (especially with 9-fold higher levels of BRAF). In particular, we already know that the V600E mutation induces a different conformation of BRAF that increases the dependency on folding machinery (CDC37/HSP90). I imagine overexpression is only going to enhance this dependency. The validation of some of the drug observations in the SBcl2 cell line and MEFs is nice, but I feel it would be beneficial to see comparison of cells lines with "normal" BRAF WT or V600E expression levels to the current overexpression system earlier in the paper (Figure 2-4).

We fully agree that it is critical to show that endogenous expression levels lead to the same/similar results as overexpression experiments and we intended to show this in the original Figure 6, but given the amount of new data, we restructured the manuscript slightly. In essence, to strengthen the point that endogenous expression levels lead to the same/similar results as overexpression experiments, we performed, as suggested, a detailed SEC-PCP-SILAC analysis using the MEF system with endogenous expression levels (see **new Figures 3 and 4**). We chose MEFs as their bi-allelic *Braf* knock-in alteration allows switching from "WT only" to "V600E only" cells, better highlighting the differences in complex distribution. To the best of our knowledge, there are no human tumour cell lines available that exclusively express $BRAF^{V600E}$, as oncogenic BRAF mutations are acting in a dominant manner. Thus, we would always have a mixture of V600E and WT complexes in tumour derived cell lines, making analyses challenging.

As observed in the ectopic expression system, we also identify the HMW and LMW complexes in MEFs, which differ in their abundance depending on V600E and WT expression. Importantly, interaction partners that we found to differentially localize to the two complex populations in the CaCo-2tet ectopic expression system, e.g. 14-3-3s, Hsp90/Cdc37, display very similar profile distribution in endogenous BRAF complexes in the MEF system. We do detect minor differences between MEFs and CaCo-2tet cells, e.g. in terms of the breadth of the overlapping SEC peak profiles, which could be cell line or organism dependent. For example, CaCo-2tet cells not only express either HA-tagged $BRAF^{WT}$ or $BRAF^{V600E}$, but still endogenous BRAF, which could affect the profiles. However, we feel that our MEF approach further supports the generality of our findings as we were able to reproduce the profiles of BRAF complexes in a distinct species.

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2nd Editorial Decision 28 November 2016

Thanks for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by the three referees. As you can see the referees appreciate the introduced revisions and support publication here. So this is the time to celebrate - and congratulations on a nice study!

Before I can send you the formal acceptance letter there are just a few things to sort out. The referees have a few remaining minor comments that are easy to address either in the text or point-by-point response.

When you submit your final version please also take care of the following points:

We encourage the publication of source data, particularly for electrophoretic gels and blots. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files

We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Can you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

It would also be good if you could provide me with a summary figure that I can place in the synopsis. The size should be 550 wide by 400 high (pixels). The model figure works - I don't know if it fits size wise.

Mass spec data should be deposited in appropriate database and the accession number provided in the main text.

The Materials and Methods section in the main text is very short. We don't really have a character limit so you have more space. OK to have parts of the materials and method section in the appendix but the main part should be in the article.

Supplemental tables should be labelled as dataset EV1, dataset EV2 etc. Please also fix the call outs in the text

In the appendix please drop the word supplemental and add appendix

We generally prefer that the figures are in portrait mode and not landscape (Figure 1, 5 and 7). If you look at the merged PDF file that the system has generated you can see how the figures look in portrait mode. It is a bit tight but perhaps it is good enough. Take a look and see what you think.

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That should be all - Let me know if you have any further questions.

REFEREE REPORTS

Referee #1:

The authors have performed a substantial amount if new work, that has improved the paper and strengthened the conclusions. It is now definitely acceptable for publication.
Just a small comment, which the authors may want to consider
p. 4: Drug resistance in the ERK pathway due to feedback mechanisms was first shown by Sturm et al. Science Signaling 3(153): ra90 (2010)

Referee #2:

The authors have adequately addressed my concerns.

Referee #3:

Overall, Diedrich et al. have attempted to address our major concern (which was shared by reviewer 1) that the paper originally relied too heavily on an over-expression system. To address this concern they used MEFs derived from homozygous BRAF floxed-V600E. Upon tamoxifen induction of Cre, cells expressing BRAF V600E under its endogenous promoter are generated. Upon induction they observe a nice shift of BRAF (V600E) into HMW fractions as compared to the uniduced BRAF Wt, which resides largely in LMW fractions. These experiments are a nice addition and are largely consistent with their Dox-inducible CaCo-2 system. One comment is that in the MEF system only a small side fraction of BRAF V600E appears to be co-eluting with CDC37, HSP90, and FKBP5 (which differs from their CaCo-2 results). Perhaps it is worth noting this.

Also, many of the minor comments were not addressed in the text or in the figures. The one particular comment that was addressed (regarding the small increase in pMEK comparing BRAF V600E vs Wt in the CaCo-2 system) was hard to follow the rationale. The concern was regarding why pMEK wasn't significantly higher for BRAF V600E over WT (as has been shown by several groups). The fact that they only observe a slight increase in pMEK with V600E would suggest that the degree to which these proteins are over-expressed is much higher than the 9-fold that is stated in the text.

Overall, the basic findings of their paper about discrete sub-populations of BRAF depending upon mutational status are interesting. Also, the impact inhibitors targeting different components of these subcomplexes (HSP90i, RAFi, or MEKi) have on the re-distribution of BRAF will be quite useful not only to the BRAF field but more generally as one thinks of consequences of used targeted therapies on any target.

2nd Revision - authors' response 06 December 2016

Referee #1:
The authors have performed a substantial amount if new work, that has improved the paper and strengthened the conclusions. It is now definitely acceptable for publication.

We thank the reviewer for the positive evaluation.

Just a small comment, which the authors may want to consider
p. 4: Drug resistance in the ERK pathway due to feedback mechanisms was first shown by Sturm et al. Science Signaling 3(153): ra90 (2010)

We would like to thank the reviewer for this comment and apologize for this oversight. We have now included Sturm et al. (2010) in the references accompanying the sentence on p.4.

Referee #2:

The authors have adequately addressed my concerns.

We thank the reviewer for the positive evaluation.

Referee #3:

Overall, Diedrich et al. have attempted to address our major concern (which was shared by reviewer 1) that the paper originally relied too heavily on an over-expression system. To address this concern they used MEFs derived from homozygous BRAF floxed-V600E. Upon tamoxifen induction of Cre, cells expressing BRAF V600E under its endogenous promoter are generated. Upon induction they observe a nice shift of BRAF (V600E) into HMW fractions as compared to the uniduced BRAF Wt, which resides largely in LMW fractions. These experiments are a nice addition and are largely consistent with their Dox-inducible CaCo-2 system. One comment is that in the MEF system only a small side fraction of BRAF V600E appears to be co-eluting with CDC37, HSP90, and FKBP5 (which differs from their CaCo-2 results). Perhaps it is worth noting this.

We thank the reviewer for the positive evaluation and added a respective statement on p. 11 to the text addressing the differential elution behavior of complexes in the cell models:
"Although elution profiles did not overlap completely as in the human CaCo-2 cells, BRAF binding partners showed a similar differential distribution,…"

Also, many of the minor comments were not addressed in the text or in the figures. The one particular comment that was addressed (regarding the small increase in pMEK comparing BRAF V600E vs Wt in the CaCo-2 system) was hard to follow the rationale. The concern was regarding why pMEK wasn't significantly higher for BRAF V600E over WT (as has been shown by several groups). The fact that they only observe a slight increase in pMEK with V600E would suggest that the degree to which these proteins are over-expressed is much higher than the 9-fold that is stated in the text.

We feel that we have addressed all the comments of referee #3, which were not highlighted as major or minor comments, in our original point-by point response and many of them led to additional data sets, either in the main figures or in the appendix. Regarding the comment referred to above, we would like to point out that our statement of 9-fold BRAF overexpression is based on our measurements. Secondly, we think that we have provided a potential explanation in the point-by – point response as to why the initial drastic differential in MEK and ERK phosphorylation visible at 12 h (more than 9-fold; original and revised **Figure 1B**) becomes less pronounced over time. As we had written in our original point-by-point response, this interesting observation remains an area for future studies and several feedback and robustness phenomena could be at play here. In this regard, we would like to add that Caco2 cells, despite their lack of *RAS* and *BRAF* mutations, display already a high level of ERK pathway activity. Hence, these cells might be particularly primed for the rapid induction of negative regulators that counteract a surge in MEK/ERK phosphorylation induced by oncogenic BRAF. As we fully agree with referee #3 that the low differential between the MEK/ERK pathway activity elicited by the two BRAF proteins should be addressed in the text, we have slightly modified (with modifications in blue) the main text on p.8 to comment this observation:

"Cells were treated with Dox for up to 96 hours and protein abundances were analyzed by immunoblotting (Figure 1A). After 6-12 h, HA-BRAF could be detected and cells responded with an increase in phospho-MEK and phospho-ERK levels (Figure 1B), with $BRAF^{V600E}$ inducing a faster response and a differential of more than 9-fold. Importantly, MEK levels stayed relatively constant over the timeframe of treatment. Interestingly, the differential in MEK/ERK phosphorylation elicited by $BRAF^{V600E}$ compared to $BRAF^{WT}$ became less pronounced with increasing induction time. The exact reason for this phenomenon is unknown, but could be potentially explained by the aforementioned feedback phenomena."

Overall, the basic findings of their paper about discrete sub-populations of BRAF depending upon mutational status are interesting. Also, the impact inhibitors targeting different components of these subcomplexes (HSP90i, RAFi, or MEKi) have on the re-distribution of BRAF will be quite useful not only to the BRAF field but more generally as one thinks of consequences of used targeted therapies on any target.

We would like thank the reviewer for the encouraging and supportive remarks and also for highlighting the broader implications of SEC-PCP-SILAC to study the effects of targeted therapy compounds on protein complexes.

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
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Each figure caption should contain the following information, for each panel where they are relevant:

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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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

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B- Statistics and general methods

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

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1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	n.a.
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For animal studies, include a statement about randomization even if no randomization was used.	n.a.
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Raw data was log2 transferred before statistical tests were performed and histograms plotting log2 fold change vs. Frequency (count) indicate normal distribution.
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C- Reagents

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies used in this study are from Cell Signaling Technology, MA, USA: anti-phospho-MEK1/2 (pS217/221) (# 9121L), anti-MEK1/2 (# 9122L), anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (#9101), anti-p44/42 MAPK (ERK1/2) (#9102); from Santa Cruz Biotechnology Inc., Heidelberg, Germany: anti-Ras (# 3965), anti-GAPDH (FL335) (# sc-25778), anti-BRAF (F7) (# sc-5284), anti-CDC37 (C-11) (# sc-13129), anti-pan-14-3-3 (H-8) (# sc-1657), normal mouse IgG (sc-2025); anti-HA (3F10) (# 11867423001, Roche Diagnostics GmbH, Mannheim, Germany), anti-HA (3F10) Affinity Matrix (# 11815016001, Roche); donkey Anti-Rabbit HRP (# NA9344V, GE Healthcare, Munich, Germany), sheep anti-Mouse HRP (# NA931V, GE Healthcare); goat Anti-Rat HRP (# 112-035-003, Dianova GmbH, Hamburg, Germany); anti-mouse IgG VeriBlot for IP secondary antibody (HRP) (# ab131368, Abcam, Cambridge, United Kingdom). The monoclonal Anti-B-Raf V600E (VE1) was kindly provided by Prof. A.v.Deimling and has been described by Capper et al., Acta Neuropathol. 2011 Jul;122(1):11-9. doi: 10.1007/s00401-011-0841-z.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were tested and mycoplasma negative. Caco2tet cells were generated in the Brummer laboratory and were derived from parental Caco2 cells as described previously (Fritsche-Günther et al., 2011). Parental Caco2 cells were obtained from Prof. Thomas Brabletz and were confirmed as Caco2 cells after expansion in the Brummer laboratory by the Multiplex human Cell line Authentication Test (MCA; Multiplexion, Heidelberg/Germany). Plat-E cells represent a long-term stock of the Brummer laboratory and were originally obtained from Dr. Kitamura (Tokyo). Sbcl2 cells were a kind gift of Dr. Meenhard Herlyn (Philadelphia) via Prof. Georg Häcker (Freiburg, Germany). Plat-E and Sbcl2 cells were not authenticated, but behaved as expected and described previously, e.g. double blasticidine /puromycin resistant (Plat-E cells, Morita et al. (2000)) and by displaying paradoxicalERK pathway activation (Sbcl2 cells; Röring et al., 2012)

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	No animals were directly used for the experiments reported in this study. The generation of murine embryonic fibroblasts, incl. the strain and genetic modifications of the parental generation is described under Materials & Methods. In brief, parental animals were housed in the specific pathogen-free barrier facility of the University Medical Center Freiburg according to institutional guidelines. Mice were kept under standard conditions (12-h light/dark cycle) with water and food ad libitum.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	No animals were directly used for the experiments reported in this study. The generation of murine embryonic fibroblasts were carried out in accordance with the local animal ethics committee (X-14/47H).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	n.a.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n.a.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	n.a.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n.a.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n.a.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n.a.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n.a.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	n.a.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD00458.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	n.a.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n.a.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Included in Results and Materials and Methods sections of the main text.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	n.a.

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.