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KSHV-TK is a tyrosine kinase that disrupts focal adhesions and induces Rho-mediated cell contraction

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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.)

Editor: Katherine Brown and David del Alamo

1st Editorial Decision 06 October 2011

Many thanks for submitting your manuscript to the EMBO Journal, and apologies for the time taken for the review - there was some delay in receiving the final report. However, I do now have the comments from all three referees, attached below. As you will see, the referees all recognise the potential interest in the work, but all also raise significant concerns with the study in its present form. Having considered the reports in detail and discussed the manuscript further with some of my colleagues, it is clear to us that the revisions required would go well beyond the scope of a normal revision. Our editorial policies are generally only to invite a revision on a manuscript when it is clear that the authors can deal with the necessary concerns within a reasonable time-frame, and when we are reasonably confident of a successful outcome. Since we do not find this to be the case with your manuscript, I am afraid we see little option other than to reject your work at this stage.

However, we do recognise the interest here, and we would encourage you to address the major concerns of the referees, and to resubmit a new version of the study in due course. To outline what we see to be the most important criticisms:

- Referee 2 finds the apparent identification of tyrosine kinase activity in TK to be potentially exciting, but does not think that you have provided strong enough evidence to demonstrate the TK genuinely is a tyrosine kinase. He/she makes constructive suggestions in this regard - most notably in that in vitro autophosphorylation assays would need to be done, as well as mass spec to confirm the identity of the phosphorylated residues.

- Referee 3 argues that your work does not really analyse the consequences of this apparent novel activity in the context of infection. While both he/she and we are aware of the difficulties in working with the lytic phase of KSHV, he/she again makes useful suggestions as to how this might be done. It would be essential to demonstrate that focal adhesions are modulated upon induction of lytic infection. It would also be important to analyse the consequences of this activity of TK on the viral life-cycle: while the story is still interesting if TK's effects on focal adhesions do not modulate lytic replication - given the cancer implications - it would be very valuable to know one way or the other.

- All the referees raise various issues with the cell biology - in that it is not entirely clear how TK modulates focal adhesions. Clearly, any insight you can gain into this would be valuable, but we would not see a full elucidation of this aspect of the manuscript to be essential for potential resubmission here.

I do hope that you will choose to work with these comments and to revise your manuscript for resubmission here, although I of course understand that you may choose to seek rapid publication elsewhere. I should add that any potential resubmission would have to be considered as a new submission rather than a revision - meaning that it would be re-assessed in the light of any related literature published in the intervening period, and that it would be subject to another round of stringent review - possibly also involving new referees. If you have any questions or comments about this, please don't hesitate to get in touch.

REFEREE REPORTS:

Referee #1 (Remarks):

In their manuscript entitled "KSHV thymidine kinase, a tyrosine kinase that disrupts focal adhesions and induces cell contraction", Gill et al characterize the atypical thymidine kinase from KSHV, revealing it to be an autophosphorylating protein kinase that promotes cell contraction through a ROCK-RhoA mechanism. As part of its activity, it induces a dislocation of FAK and paxillin from focal adhesions. Although several key pieces of information are still lacking (to what does KSHV-TK bind, what are it's kinase targets in addition to itself) this study defines a novel function for a TK enzyme, that could contribute to cancer-like behaviour in infected cells.

In general, the experiments are well done. However, at times they suffer from a lack of quantification and some important control experiments are lacking. My specific comments are below, which are intended to address these concerns and make this article suitable for publication.

1. Fig 2A: The authors claim that GFP-KSHV-TK overlaps with a subset of actin filaments, but this is difficult to appreciate without an overlay for comparison.

2. An important point that the authors mention is that cells increase their stress fibres before they start to contract: there is no qualitative or quantitative example of this. A movie of Lifeact-expressing cells would be useful to appreciate the cytoskeletal changes that occur prior to contraction, rounding and blebbing.

3. Fig 3: this figure suffers from a lack of quantification. Some quantitative method should be used (for example flourescence units per unit area compared to GFP controls) to determine by how much phosphorylation and/or localization is reduced in KHSV-TK-expressing cells.

4. Treatment with blebbistatin or Y-27632 prevents cell contraction (fig 2); does it have any effect on the phosphorylation status of FAK and paxillin in FAs?

5. Fig 4D: it is impossible to tell how FAKY925 is affected in this panel since the cells shown in Fig 4D and Fig 3A look quite different from one another. Staining is very bright in 4D.

6. Fig 7B: TK-DEAD does not come down in FAK pulldowns. The most likely explanation for this is that the autophosphorylation sites provide binding sites for KSHV-TK to form a complex that includes FAK. Therefore, my question: can the Y65/85F mutant be pulled down with FAK? Connected to this, the sentence in the discussion, "The ability of KSHV-TK to associate with FAK only occurs when the kinase is auto-phosphorylated on tyrosines 65 and 85," has not been shown here.

Referee #2 (Remarks):

In this paper the authors have reinvestigated the functions of the KSHV virally-encoded thymidine kinase in inducing cellular phenotypes. They started by showing that expression of KSHV-TK in HeLa cells induced cell contraction and blebbing, and used RhoN19 and Y27632/blebbistatin to show that this was dependent on Rho and ROCK-mediated actomyosin contraction. Consistent with this, GFP-KSHV-TK expressing cells exhibited elevated levels of RhoA.GTP. They went on to demonstrate that GFP-KSHV-TK expression disrupted the integrity of focal adhesions, causing decreases in total P.Tyr, and FAK pY925/paxillin pY31 levels in focal adhesions, as well as reducing the levels of FAK, paxillin and zyxin proteins. Next, they replaced the endogenous TK gene in MuHV-4 herpesvirus with GFP-KSHV-TK by recombination. Infection with MuHV-4[g-KSHV-TK] virus but not MuHV-4 itself caused disruption of focal adhesions. Interestingly, they found that GFP-KSHV-TK was phosphorylated on tyrosine in MuHV-4[g-KSHV-TK] infected cells, and that a TK-dead mutant GFP-KSHV-TK had greatly reduced levels of P.Tyr, and also failed to induce cellular morphology changes and decrease pY925 FAK and pY31 paxillin levels. Tyr65 andTyr85 were identified as GFP-KSHV-TK Tyr phosphorylation through an N-terminal deletion series and individual Tyr to Phe point mutations. The Y65/85F GFP-KSHV-TK mutant failed to induced morphological changes, and, like GFP-KSHV-TK-DEAD, GFP-KSHV-TK, Y65/85F failed to increase Rho.GTP levels. Finally, they showed that GFP-KSHV-TK expression reduced the association of FAK with paxillin but did not affect FAK association with c-Src. Moreover, WT but not TK-DEAD GFP-KSHV-TK coprecipitated with FAK, and siRNA-mediated depletion of paxillin was found to decrease the ability of GFP-KSHV-TK to cause morphological changes or loss of proteins from focal adhesions.

The conclusion that KSHV-TK has an autophosphorylating activity that leads to phosphorylation of two Tyr in its N-terminal region, and that these phosphorylated Tyr cause dramatic cytoskeletal and focal adhesion morphology changes in infected cells is intriguing, and suggests that KSHV TK may have two distinct functions in the infected cell. However, this is the first time that GFP-KSHV-TK has been reported to be phosphorylated on Tyr, and more rigorous evidence is required to establish this unequivocally. For instance, the authors need to do additional experiments to demonstrate that the anti-P.Tyr MAb PY99 signal is really due to P.Tyr, e.g. treatment with recombinant PTP. Moreover, the sites of Tyr phosphorylation were identified only indirectly by mutations, and some direct evidence that Tyr65 and Tyr85 are phosphorylated would be reassuring (e.g. MS data or sequence-specific antibodies to P.Tyr65 or P.Tyr85). In addition, the fact that mutation of these two Tyr to Phe abolishes the ability of KSHV to induce morphology changes does not strictly mean that this is due to a lack of phosphorylation of these residues, because there could simply be a requirement for the OH groups on these two Tyr.

The other main weakness is that there are no true mechanistic insights into how these two Tyr are autophosphorylated, and there are a number of questions in this regard: does autophosphorylation occur cis or in trans; can the isolated catalytic domain phosphorylate the N-terminal fragment in trans or any other substrate protein; and, are these two Tyr the only residues phosphorylated in KSHV TK - Ser and Thr residues could also be phosphorylated- an MS/MS analysis of the isolated KSHV TK protein would be revealing. Most importantly, in order to establish that these Tyr are autophosphorylated by an activity intrinsic to KSHV TK rather than being phosphorylated by

another tyrosine kinase in the cell (this is a real possibility given the association of KSHV with FAK/Src - see point 6) in vitro phosphorylation experiments with purified bacterially-expressed WT and DEAD KSHV TK protein are essential. This would also allow the authors to test if KSHV-TK can phosphorylate other proteins and with what specificity.

The other major unanswered question is how P.Tyr65/P.Tyr85 are able to induce the observed cytoskeletal changes; do they bind to SH2/PTB domain proteins or are they acting in some other way; how does KSHV TK associate with FAK (what FAK domain is involved, and can the phosphorylated N-terminal fragment of KSHV-TK or full-length KSHV-TK bind FAK in vitro?), and how does KSHV-TK displace paxillin from binding to the FAK FAT domain; and, why does MuHV-4 TK not causes these effects if it can also be phosphorylated on Tyr?

Other points: 1. Figure 2: There was significant cell-to-cell variability in the level of expression of GFP-KSHV-TK, and it is not clear that the cell morphology and the actin cytoskeletal phenotypes actually correlated very well with the levels of GFP-KSHV-TK expression.

2. Figure 3: The GFP-KSHV-TK-expressing cells of interest need to be labeled with arrowheads in the GFP panel as well as in the protein staining panel of the same cells. Can decreases in pY925 FAK and pY31 paxillin be observed by immunoblotting whole cell proteins, or by immunoblotting FAK and paxillin immunoprecipitates? Is there a change in the total level of paxillin and zyxin upon KSHV-TK expression (this is not clear from Figure 7A)?

3. Figure 4B: It is unclear from the legend and the text exactly what was done here, and MuHV-4 Revert is not properly described, forcing the reader to look up Coleman et al. (2003) to find out what it is. One assumes that these are immunoblots of whole cell proteins with PY99 and anti-GFP. If these are whole cell protein blots, then one cannot be certain that the P.Tyr-positive band in the MuHV-4[g-KSHV-TK] lane is in fact GFP-KSHV-TK, and the authors need to isolate the GFP-KSHV-TK protein by immunoprecipitating with anti-GFP antibodies and then blot this with anti-P.Tyr antibodies, using a PTP-treated sample as a control. Is the band in the MuHV-4 and MUHV-4 Revert lanes P.Tyr-containing MuHV-4 TK? If this the case, why does MuHV-4 TK not induce changes in cell morphology? This issue is not discussed, and raises the question of what is special about KSHV-TK compared to MuHV-4 TK. Based on a cursory sequence comparison of the KSHV and MuHV-4 TKs, the two Tyr appear to be conserved, and the sequences around "Y65" are quite similar. Finally, the whole gel needs to be shown to demonstrate how the global P.Tyr pattern in the infected cells was changed (this would also need an uninfected cell control).

4. Figures 4 and 5: Can a dissociation-of-function catalytic domain mutant be made that lacks autophosphorylating activity but retains TK activity (and vice versa)?

5. Figure 6: Based on these data, it appears that Y65F and Y85F KSHV-TK are both able to induce morphology changes, whereas the Y65/85F mutant cannot. This implies that pY65 and pY85 have redundant functions even though the sequence around them is very different.

6. Figure 7: The authors have deduced that KSHV-TK is autophosphorylated in vivo, because the KSHV-TK-DEAD mutant shows reduced P.Tyr content. However, since GFP-KSHV-TK-DEAD does not associate with FAK/Src, it is possible that GFP-KSHV-TK is in fact not autophosphorylated but rather is phosphorylated in trans by FAK or Src. Does the association of KSHV with FAK require phosphorylation of Tyr65/85 - the authors need to test this with the F65/85 GFP-KSHV-TK mutant. Does MuHV-4 TK associate with FAK?

7. Figure 7D and E: The authors state that depletion of paxillin did not affect Tyr phosphorylation of KSHV TK but do not show this directly. It is somewhat surprising that depletion of paxillin per se did not have a more dramatic effect on focal adhesion morphology.

Referee #3 (Remarks):

This group has previously shown that KSHV TK does not have strong TK activity and induces cell shape changes. In this manuscript they begin to study the cell shape changes induced by KSHV TK. They find that KSHV-TK induces cell contraction dependent on ROCK and RhoA and there is a decreased expression of surface focal adhesions along with other changes associated with focal adhesions like paxillin. They also find that tyrosine phosphorylation of KSHV-TK is necessary for these effects. They replace the TK of MuHV-4 with the TK of KSHV and find that during lytic infection in cell culture they see similar effects as they saw with the KSHV-TK alone but they do not see these effects with the natural MuHV-4 TK. While the phenotypic effects of the KSHV-TK are interesting and provide evidence for functions other than a thymidine kinase for the KSHV protein, there is little discussion of the role of this effect in KSHV biology or lytic replication and there is no evidence that this might be important for KSHV replication or any part of KSHV biology. Also, they spend a great deal of the introduction and discussion stating that for understanding how antivirals that work against TK work we need to understand all the functions of TK. While the other functions are definitely of interest, only an understanding of the thymidine kinase activity is needed, other functions play other roles and are unrelated to the antivirals. There are also some places in the manuscript that need additional data to clearly make their points. These are listed below.

1. Overall, the effects found are an interesting phenomenon but the actual outcome of these effects for the virus are not discussed. There is no evidence that KSHV lytic replication leads to these effects. While they state there is no lytic system for KSHV, there are ways to induced KSHV lytic replication in a sub-set of cells, and through their microscopy, they could identify these and determine if there is a change in the focal adhesions or paxillin etc... This might provide some meaning to the phenomenon they describe.

To be more specific, the authors should induce lytic replication in infected cells with Rta (expressed from a transfected plasmid or adenovirus as has been described in the literature) and the block the downstream changes induced by KSHV TK using the drugs described or the siRNA to paxillin. They could then quantify the virus released and thereby determine if the KSHV-TK plays a critical role in lytic replication of KSHV. This would provide relevance of this interesting phenomenon they describe.

2. In figure 2 they describe co-localization of KSHV-TK with Actin stress fibers. This is very difficult to see without an overlay picture with different colors as the TK is all over the cell and the Actin fibers are widely distributed.

3. In figure 3 they describe the disassembly and loss of phosphorylation of focal adhesions and FAK. They would enhance this loss with a more quantitative western blot analysis of phosph-FAK.

4. In figure 4D it looks like p-FAK is increased all over the cytoplasm in the presence of KSHV-TK. What is this localization and increase in phsopho-FAK?

5. What is the logic that to understand the ability of antivirals to work against KSHV, all the functions of TK must be known. Knowing that the KSHV TK does not have strong thymidine kinase activity leads to the understanding of why some antivirals don't work. While the other functions may be interesting, they do not shed light on the kinase activity per se.

Resubmission 14 October 2013

Referee #1 (Remarks to the Author):

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In general, the experiments are well done. However, at times they suffer from a lack of quantification and some important control experiments are lacking. My specific comments are below, which are intended to address these concerns and make this article suitable for publication.

1. Fig 2A: The authors claim that GFP-KSHV-TK overlaps with a subset of actin filaments, but this is difficult to appreciate without an overlay for comparison.

We have now included an additional set of images showing individual actin and GFP-KSHV-TK channels as well as a merged image. These images clearly show that GFP-KSHV-TK filaments aligning with actin stress fibres.

2. An important point that the authors mention is that cells increase their stress fibres before they start to contract: there is no qualitative or quantitative example of this. A movie of Lifeact-expressing cells would be useful to appreciate the cytoskeletal changes that occur prior to contraction, rounding and blebbing.

We have now provided the requested quantification at 16h post transfection in Figure S2A since during this time frame the cells undergo the mots pronounced changes in morphology. This quantification clearly shows that GFP-KSHV-TK but not GFP alone induces actin stress fibres prior to cell rounding.

3. Fig 3: this figure suffers from a lack of quantification. Some quantitative method should be used (for example flourescence units per unit area compared to GFP controls) to determine by how much phosphorylation and/or localization is reduced in KHSV-TK-expressing cells.

We have now quantified the total cell intensity for phospho-paxillin in GFP and GFP-KSHV-TK expressing cells as requested. Representative images are shown in Figure S3B and the drop in fluorescence intensity has been added to the main text on page 7.

4. Treatment with blebbistatin or Y-27632 prevents cell contraction (fig 2); does it have any effect on the phosphorylation status of FAK and paxillin in FAs?

Treating cells with either drug in the absence of KSHV-TK does not alter the staining of phoshotyrosine, phospho-PaxillinY31 or phospho-FAKY925 at focal adhesions. We have included an example of these data in Figure S2B.

5. Fig 4D: it is impossible to tell how FAKY925 is affected in this panel since the cells shown in Fig 4D and Fig 3A look quite different from one another. Staining is very bright in 4D.

We agree that comparing the effects of KSHV-TK on focal adhesions in Fig. 3A (transfected GFP-KSHV-TK) and 4D (virally expressed GFP-KSHV-TK) is not so clear. It is also not a like for like experiment given the different expression methods. We have now replaced panel D and E in figure 4 in our original manuscript with a single panel (now panel D) that compares the staining for focal adhesion proteins in cells infected with the parental MuHV-4 or the recombinant MuHV-4 expressing GFP-KSHV-TK. We feel this comparison of the effects of the presence or absence of GFP-KSHV-TK in MuHV-4 infected cells makes it easier to see the direct impact of GFP-KSHV-TK on focal adhesion integrity.

6. Fig 7B: TK-DEAD does not come down in FAK pulldowns. The most likely explanation for this is that the autophosphorylation sites provide binding sites for KSHV-TK to form a complex that includes FAK. Therefore, my question: can the Y65/85F mutant be pulled down with FAK? Connected to this, the sentence in the discussion, "The ability of KSHV-TK to associate with FAK only occurs when the kinase is auto-phosphorylated on tyrosines 65 and 85," has not been shown here.

We have now included an IP experiment and show that the Y65/85F and Y65/85/120F mutants maintain their association with FAK (show in Figure 7A-B). We have also stated in the discussion that the although both the Tyrosine 65 and 85 are required to induce the focal adhesion disassembly observed when expressing the WT protein there must be an additional protein(s) that link TK through FAK to intracellular signalling pathways (Discussion, Page 12, Paragraph 2). Consistent with the data presented in our original submission, in contrast to both WT and Tyrosine mutants of KSHV-TK (Y65/85F and Y65/85/120F) which retain their ability to associated with FAK, the KINASE-DEAD mutant does not show any association with FAK. Therefore we can show that KSHV-TK only interacts with FAK when it retains its kinase activity.

Referee #2 (Remarks to the Author):

In this paper the authors have reinvestigated the functions of the KSHV virally-encoded thymidine kinase in inducing cellular phenotypes. They started by showing that expression of KSHV-TK in HeLa cells induced cell contraction and blebbing, and used RhoN19 and Y27632/blebbistatin to show that this was dependent on Rho and ROCK-mediated actomyosin contraction. Consistent with this, GFP-KSHV-TK expressing cells exhibited elevated levels of RhoA.GTP. They went on to demonstrate that GFP-KSHV-TK expression disrupted the integrity of focal adhesions, causing decreases in total P.Tyr, and FAK pY925/paxillin pY31 levels in focal adhesions, as well as reducing the levels of FAK, paxillin and zyxin proteins. Next, they replaced the endogenous TK gene in MuHV-4 herpesvirus with GFP-KSHV-TK by recombination. Infection with MuHV-4[g-KSHV-TK] virus but not MuHV-4 itself caused disruption of focal adhesions. Interestingly, they found that GFP-KSHV-TK was phosphorylated on tyrosine in MuHV-4[g-KSHV-TK] infected cells, and that a TK-dead mutant GFP-KSHV-TK had greatly reduced levels of P.Tyr, and also failed to induce cellular morphology changes and decrease pY925 FAK and pY31 paxillin levels. Tyr65 andTyr85 were identified as GFP-KSHV-TK Tyr phosphorylation through an N-terminal deletion series and individual Tyr to Phe point mutations. The Y65/85F GFP-KSHV-TK mutant failed to induced morphological changes, and, like GFP-KSHV-TK-DEAD, GFP-KSHV-TK, Y65/85F failed to increase Rho.GTP levels. Finally, they showed that GFP-KSHV-TK expression reduced the association of FAK with paxillin but did not affect FAK association with c-Src. Moreover, WT but not TK-DEAD GFP-KSHV-TK coprecipitated with FAK, and siRNA-mediated depletion of paxillin was found to decrease the ability of GFP-KSHV-TK to cause morphological changes or loss of proteins from focal adhesions.

The conclusion that KSHV-TK has an autophosphorylating activity that leads to phosphorylation of two Tyr in its N-terminal region, and that these phosphorylated Tyr cause dramatic cytoskeletal and focal adhesion morphology changes in infected cells is intriguing, and suggests that KSHV TK may have two distinct functions in the infected cell. However, this is the first time that GFP-KSHV-TK has been reported to be phosphorylated on Tyr, and more rigorous evidence is required to establish this unequivocally. For instance, the authors need to do additional experiments to demonstrate that the anti-P.Tyr MAb PY99 signal is really due to P.Tyr, e.g. treatment with recombinant PTP. Moreover, the sites of Tyr phosphorylation were identified only indirectly by mutations, and some direct evidence that Tyr65 and Tyr85 are phosphorylated would be reassuring (e.g. MS data or sequence-specific antibodies to P.Tyr65 or P.Tyr85). In addition, the fact that mutation of these two Tyr to PHe abolishes the ability of KSHV to induce morphology changes does not strictly mean that this is due to a lack of phosphorylation of these residues, because there could simply be a requirement for the OH groups on these two Tyr.

The other main weakness is that there are no true mechanistic insights into how these two Tyr are autophosphorylated, and there are a number of questions in this regard: does autophosphorylation occur cis or in trans; can the isolated catalytic domain phosphorylate the N-terminal fragment in trans or any other substrate protein; and, are these two Tyr the only residues phosphorylated in KSHV TK - Ser and Thr residues could also be phosphorylated- an MS/MS analysis of the isolated KSHV TK protein would be revealing. Most importantly, in order to establish that these Tyr are autophosphorylated by an activity intrinsic to KSHV TK rather than being phosphorylated by another tyrosine kinase in the cell (this is a real possibility given the association of

KSHV with FAK/Src - see point 6) in vitro phosphorylation experiments with purified bacterially-expressed WT and DEAD KSHV TK protein are essential. This would also allow the authors to test if KSHV-TK can phosphorylate other proteins and with what specificity.

The reviewer has raised a number of very important points that we have done our best to address.

1. We have shown that in contrast to the TK's from the gammaherpesviruses EBV and MuHV-4, the KSHV-TK is autophosphorylated when expressed in *E. coli*. Moreover, this activity is dependent on its kinase domain and not an *E. coli* kinase, as the Kinase dead GST-KSHV-TK mutant is not phosphorylated.

2. We have performed mass spec analysis of GST-KSHV-TK purified from *E. coli*. This analysis confirms that tyrosine 65 and 85 are phosphorylated by the KSHV-TK kinase domain (see Fig. S7). In addition, this analysis has also shown that tyrosine 120 is also phosphorylated. We have looked at the impact of the triple tyr mutant on Rho activation, its ability to associate with FAK/Paxillin, focal adhesion integrity and cell morphology.

3. We have examined whether the C-terminal kinase domain can phosphorylate the N-terminal half of KSHV-TK when co-expressed in cells. The data in Fig. 5G shows that phosphorylation only occurs in the intact full-length protein and not in trans.

The other major unanswered question is how P.Tyr65/P.Tyr85 are able to induce the observed cytoskeletal changes; do they bind to SH2/PTB domain proteins or are they acting in some other way; how does KSHV TK associate with FAK (what FAK domain is involved, and can the phosphorylated N-terminal fragment of KSHV-TK or full-length KSHV-TK bind FAK in vitro?), and how does KSHV-TK displace paxillin from binding to the FAK FAT domain; and, why does MuHV-4 TK not causes these effects if it can also be phosphorylated on Tyr?

MuHV-4-TK and KSHV-TK do show sequence conservation around tyrosine residues 65 and 85. Tyrosine residue 120 in KSHV-TK shows limited sequence conservation in MuHV-4-TK. However, we have no idea which tyrosine residue(s) are phosphorylated in MuHV-4-TK. The kinase domain of MuHV-4-TK is clearly not a tyrosine kinase. Consistent with this, the tyrosine phopshorylation of MuHV-4-TK is dependent on Src family kinases as it is inhibited by PP2 and lacks phosphorylation when expressed in a Src/Fyn/Yes deficient cell line ($SYF^{-/-}$). The phosphorylation of MuHV-4-TK is also rescued when it is co-expressed with SRC-GFP in the $SYF^{-/-}$ (MBG unpublished observations/manuscript in preparation). We are happy to provide this supportive data to the referees if required for confirmation. This does not rule out that the equivalent tyrosines in MuHV-4-TK are not phosphorylated albeit by host Src family kinases. However, our pull down data with KSHV-TK and its kinase dead and triple tyr mutants clearly shows that its association with FAK/Paxillin is dependent on its tyrosine kinase activity and not its autophosphorylation. MuHV-4-TK expression has no impact upon focal adhesion integrity and cell morphology, in contrast to that observed when expressing KSHV-TK.

Moreover, our data clearly show that KSHV-TK mediated disruption of focal adhesion integrity and induction of cell rounding is dependent on both its autophosphorylation and its kinase domain, based on the absence of a phenotype with the kinase dead and triple tyrosine mutants. These two mutants, however, have different impacts on the association of KSHV-TK with FAK and paxillin. The most straightforward interpretation is that disruption of focal adhesion integrity and induction of cell rounding is dependent on autophosphorylation of KSHV-TK and phosphorylation of addition cellular targets. Identification of these additional targets will undoubtedly be important to understand how KSHV-TK exerts its effects. However, at this stage we feel that it is beyond the scope of the current study, which has already provided important new insights into the activity of KSHV-TK, namely it is a tyrosine kinase.

We have not further analysed the specific domain interaction between FAK and KSHV-TK in this study since both the double and triple tyrosine mutants of KSHV-TK retain their ability to interact with FAK. Therefore we recognise that an additional protein(s) is required to promote KSHV-TK induce focal adhesion disassembly beyond the requirement of FAK. The identification and characterisation of KSHV-TK interacting proteins is part of our ongoing and mechanistic studies to determine precisely how KSHV-TK impacts upon cell signalling pathways.

Other points: 1. Figure 2: There was significant cell-to-cell variability in the level of expression of GFP-KSHV-TK, and it is not clear that the cell morphology and the actin cytoskeletal phenotypes actually correlated very well with the levels of GFP-KSHV-TK expression.

The level of expression of GFP-KSHV-TK does vary from cell to cell. A similar variation is also seen in GFP alone, which is not unexpected in transient expression experiments. However, there does not appear to be a correlation between expression level and phenotype as even low levels of GFP-KSHV-TK still induce cell rounding, presumably as it is a potent tyrosine kinase that is not regulated/inhibited by the cell.

We have now also provided quantification of changes in actin stress fibres and cell rounding in cells expressing GFP and GFP-KSHV-TK in Figure S2A. We hope this data addresses the reviewers concerns.

2. Figure 3: The GFP-KSHV-TK-expressing cells of interest need to be labeled with arrowheads in the GFP panel as well as in the protein staining panel of the same cells. Can decreases in pY925 FAK and pY31 paxillin be observed by immunoblotting whole cell proteins, or by immunoblotting FAK and paxillin immunoprecipitates? Is there a change in the total level of paxillin and zyxin upon KSHV-TK expression (this is not clear from Figure 7A)?

We have added arrowheads as requested. We have tried to look at the levels of pY925 FAK and pY31 paxillin by immunoblot. In our hands the antibodies have not worked well or consistently for immunoblotting. Given our transfection efficiencies are not 100% we have quantified the levels of pY925 FAK and pY31 paxillin at focal adhesions (see reviewer 1). This analysis reveals that the levels of

phosphorylation of FAK and paxillin are reduced in GFP-KSHV-TK expressing cells (Fig. S3B). To further address this reduction by immunoblot analysis we have immunoprecipitated FAK and Paxillin in both GFP and GFP-KSHV-TK expressing cells and blotted the precipitates for pan phosphotyrosine. A reduction in both the tyrosine phosphorylation of FAK and Paxillin in KSHV-TK expressing cells is now shown in Figure S3B.

3. Figure 4B: It is unclear from the legend and the text exactly what was done here, and MuHV-4 Revert is not properly described, forcing the reader to look up Coleman et al. (2003) to find out what it is. One assumes that these are immunoblots of whole cell proteins with PY99 and anti-GFP. If these are whole cell protein blots, then one cannot be certain that the P.Tyr-positive band in the MuHV-4[g-KSHV-TK] lane is in fact GFP-KSHV-TK, and the authors need to isolate the GFP-KSHV-TK protein by immunoprecipitating with anti-GFP antibodies and then blot this with anti-P.Tyr antibodies, using a PTP-treated sample as a control. Is the band in the MuHV-4 and MUHV-4 Revert lanes P.Tyr-containing MuHV-4 TK? If this the case, why does MuHV-4 TK not induce changes in cell morphology? This issue is not discussed, and raises the question of what is special about KSHV-TK compared to MuHV-4 TK. Based on a cursory sequence comparison of the KSHV and MuHV-4 TKs, the two Tyr appear to be conserved, and the sequences around "Y65" are quite similar. Finally, the whole gel needs to be shown to demonstrate how the global P.Tyr pattern in the infected cells was changed (this would also need an uninfected cell control).

We have modified Figure 4 for further clarity. We have removed the data for the Revertant and TK- viruses since they did not add anything to the main message of the paper. The recombinant MuHV-4 virus expressing GFP-KSHV-TK was generated using standard BAC mutagenesis as detailed in the materials and methods. We now show cells infected with WT versus GFP-KSHV-TK expressing recombinant virus to allow a direct comparison to be made (Fig. 4C). We have also included complete blots to show all changes to the global levels of phosphotyrosine (Fig. 4B) as requested. It is clear that the predominant proteins modified by tyrosine phosphorylation are the viral encoded TKs. The phosphotyrosine band for GFP-KSHV-TK corresponds to the GFP-positive signal. The identity of the GFP-positive protein was confirmed as GFP-KSHV-TK by mass spec analysis of a anti-GFP immuno-precipitation. This data has not been included in the paper but can be provided if the reviewer feels it is necessary.

The fact that MuHV-4-TK does not induce changes in focal adhesion integrity and cell rounding has already been discussed above in relation to an earlier question raised by the reviewer.

4. Figures 4 and 5: Can a dissociation-of-function catalytic domain mutant be made that lacks autophosphorylating activity but retains TK activity (and vice versa)?

This is a very interesting idea. However, at this point it will be difficult to addess since KSHV-TK has almost undetectable Thymidine kinase activity when assayed using conventional TK assays (Gustafson EA et al., J Virol. 2000, 74: 684–692.). We also feel it will be easier to address once we have some structural data.

5. Figure 6: Based on these data, it appears that Y65F and Y85F KSHV-TK are both able to induce morphology changes, whereas the Y65/85F mutant cannot. This implies that pY65 and pY85 have redundant functions even though the sequence around them is very different.

From our additional studies we have also identified an additional tyrosine residue at amino acid residue 120 that is auto-phosphoyrlated in KSHV-TK. Unlike the expressed WT protein which induces disassemby of focal adhesions the expression of the double (Y65/85) and triple (Y65/85/120F) tyrosine mutants fail to induce cellular remodelling. With the triple mutant showing a phenotype almost identical to the mock transfected cells- flattened morphology and normal assembly of focal adhesions. It is feasible that in the context of the full protein the three tyrosine are used to interact with a SH2 domains of neighbouring proteins (or one protein with two tandem SH2 domains). It would be interesting to examine what cellular protein(s) bind the individual phosphorylated tyrosines but at this stage we feel that's one for the future and beyond the papers scope.

6. Figure 7: The authors have deduced that KSHV-TK is autophosphorylated in vivo, because the KSHV-TK-DEAD mutant shows reduced P.Tyr content. However, since GFP-KSHV-TK-DEAD does not associate with FAK/Src, it is possible that GFP-KSHV-TK is in fact not autophosphorylated but rather is phosphorylated in trans by FAK or Src. Does the association of KSHV with FAK require phosphorylation of Tyr65/85 - the authors need to test this with the F65/85 GFP-KSHV-TK mutant. Does MuHV-4 TK associate with FAK?

KSHV-TK can be expressed in FAK- and Src- (Yes and Fyn) deficient SYF cells and is still tyrosine phosphorylated. More importantly, KSHV-TK expressed and purified from *E. coli* is phosphorylated on three tyrosine residues (see Figure 5F for immunoblots and Figure S7 for MS/MS analysis). This is in contrast to MuHV-4 TK which we now know is phosphorylated by cellular enzymes (Src/Fyn and Yes). The tyrosine phopshorylation of MuHV-4-TK is dependent on Src family kinases as it is inhibited by PP2 and lacks phosphorylation when expressed in a Src/Fyn/Yes deficient cell line ($SYF^{-/-}$). The phosphorylation of MuHV-4-TK is also rescued when it is co-expressed with SRC-GFP in the $SYF^{-/-}$ (MBG unpublished observations/manuscript in preparation). We are happy to provide this supportive data to the referees if required for confirmation.

7. Figure 7D and E: The authors state that depletion of paxillin did not affect Tyr phosphorylation of KSHV TK but do not show this directly. It is somewhat surprising that depletion of paxillin per se did not have a more dramatic effect on focal adhesion morphology.

Paxillin depletion increases Rac1 activity (*) and will maintain the cell in a more ruffled and flattened morphology. Therefore the expression of KSHV-TK a protein that normal induces cell rounding will be counteracted by the depletion of Paxillin.
(*)Nicholas O. Deakin and Christopher E. Turner. **Distinct roles for paxillin and Hic-5 in regulating breast cancer cell morphology, invasion, and metastasis.** Mol Biol Cell. 2011. 22(3): 327–341.

Referee #3 (Remarks to the Author):

This group has previously shown that KSHV TK does not have strong TK activity and induces cell shape changes. In this manuscript they begin to study the cell shape changes induced by KSHV TK. They find that KSHV-TK induces cell contraction dependent on ROCK and RhoA and there is a decreased expression of surface focal adhesions along with other changes associated with focal adhesions like paxillin. They also find that tyrosine phosphorylation of KSHV-TK is necessary for these effects. They replace the TK of MuHV-4 with the TK of KSHV and find that during lytic infection in cell culture they see similar effects as they saw with the KSHV-TK alone but they do not see these effects with the natural MuHV-4 TK. While the phenotypic effects of the KSHV-TK are interesting and provide evidence for functions other than a thymidine kinase for the KSHV protein, there is little discussion of the role of this effect in KSHV biology or lytic replication and there is no evidence that this might be important for KSHV replication or any part of KSHV biology. Also, they spend a great deal of the introduction and discussion stating that for understanding how antivirals that work against TK work we need to understand all the functions of TK. While the other functions are definitely of interest, only an understanding of the thymidine kinase activity is needed, other functions play other roles and are unrelated to the antivirals. There are also some places in the manuscript that need additional data to clearly make their points. These are listed below.

1. Overall, the effects found are an interesting phenomenon but the actual outcome of these effects for the virus are not discussed. There is no evidence that KSHV lytic replication leads to these effects. While they state there is no lytic system for KSHV, there are ways to induced KSHV lytic replication in a sub-set of cells, and through their microscopy, they could identify these and determine if there is a change in the focal adhesions or paxillin etc... This might provide some meaning to the phenomenon they describe.

To be more specific, the authors should induce lytic replication in infected cells with Rta (expressed from a transfected plasmid or adenovirus as has been described in the literature) and the block the downstream changes induced by KSHV TK using the drugs described or the siRNA to paxillin. They could then quantify the virus released and thereby determine if the KSHV-TK plays a critical role in lytic replication of KSHV. This would provide relevance of this interesting phenomenon they describe.

2. In figure 2 they describe co-localization of KSHV-TK with Actin stress fibers. This is very difficult to see without an overlay picture with different colors as the TK is all over the cell and the Actin fibers are widely distributed.

We have included a merged image here to show how the TK and actin can run parallel to each other.

3. In figure 3 they describe the disassembly and loss of phosphorylation of focal adhesions and FAK. They would enhance this loss with a more quantitative western blot analysis of phosph-FAK.

We have included an extra panel in Supplemental data to show the corrected total cell fluorescence to demonstrate the reduction in phospho-Paxillin Y31-

demonstrating the decrease in total cell fluorescence intensity in cells expressing KSHV-TK when compared to those expressing GFP (Figure S3B).

4. In figure 4D it looks like p-FAK is increased all over the cytoplasm in the presence of KSHV-TK. What is this localization and increase in phsopho-FAK?

Phospho-FAK appears to be more cytoplasmic in KSHV-TK expressing cells. Since the both proteins associate the TK might be sequestering this focal adhesion protein in the cytoplasm and preventing its trafficking or recruitment to focal adhesions.

5. What is the logic that to understand the ability of antivirals to work against KSHV, all the functions of TK must be known. Knowing that the KSHV TK does not have strong thymidine kinase activity leads to the understanding of why some antivirals don't work. While the other functions may be interesting, they do not shed light on the kinase activity per se.

If the KSHV-TK can act as a tyrosine kinase then this is a powerful way for the virus and one that is separate from its function as a TK to modulate the normal function of the cell. Cells tightly regulate tyrosine phosphorylation of proteins as this modification can dramatically affect the activity of a protein leading to changes in a multitude of cellular processes including cell signalling and migration. Indeed, aberrant activation or mutation of Src/Abl and FAK kinases is a hallmark of cell transformation and tumour development and invasion. Therefore in a quiescent "normal" cell the presence of phosphorylated KSHV-TK as well as its ability to tyrosine phosphorylate host proteins is likely to lead to the activation and/or inhibition of multiple signalling pathways regulating essential cellular processes such as adhesion and migration. Thus it is important to understand the tyrosine kinase activity of KSHV-TK to help faciliate identifying drugs that will inhibit its activity as they may be useful to treat diseases associated with lytically replicating KSHV.

2nd Editorial Decision 17 October 2013

Thank you for the re-submission of your research manuscript entitled "KSHV-TK is a tyrosine kinase that disrupts focal adhesions and induces Rho-mediated cell contraction". I have now evaluated it and discussed its suitability within our editorial team according to the scope and aim of The EMBO Journal.

We certainly appreciate that the resubmitted manuscript has seen some improvements in addressing the concerns raised in the prior version, more specifically regarding the demonstration that KSHV TK is actually a tyrosine kinase. However, we believe that most of the concerns raised by the referees, including those mentioned by my former colleague Katherine Brown in her original rejection letter as required for further consideration of your study, have not been properly addressed. This applies in particular to the physiological role of KSHV TK in the viral life cycle as explicitly formulated by referee #3. With these concerns in mind, I am afraid that we cannot offer to continue with the publication process of your manuscript.

I am sorry to disappoint you again and I hope for the rapid publication of your manuscript somewhere else.

Appeal 23 October 2013

Thank you for your recent letter concerning our MS (EMBOJ-2013-87154), although after such an extensive revision we are obviously disappointed with the outcome. Having read your concerns carefully, we felt it was necessary to provide further clarity as you why more specific research was not undertaken to address the concerns of referee 3 from our original submission.

If we were trying to ascertain the global contribution of KSHV-TK to lytic viral replication, i.e. not separating out its newly identified tyrosine kinase activity from its thymidine kinase activity, the experiments suggested by referee 3 would be an excellent approach. However, our manuscript was focused on highlighting the novel and unexpected tyrosine kinase activity of this viral nucleoside kinase rather than analyse its requirement for viral lytic replication.

Proposed experiments:

1. Inducing lytic replication of KSHV and examining the impact upon focal adhesions via the staining of specific FA proteins (FAK and Paxillin).

This is a good experiment that we'd love to be able to do. However, to fully evaluate the true contribution of the novel tyrosine kinase activity of KSHV-TK, and its ability to modulate focal adhesion dynamics, one would have to generate a recombinant KSHV in which the endogenous TK is replaced by the triple Tyrosine mutant (KSHV-TK-Y65/85/120F). Unfortunately at the moment it is well recognized in the field of KSHV research that the published KSHV BAC systems for generating recombinant viruses are flawed. Having already successfully generated KSHV BAC to specifically address a potential reviewer concern (as highlighted by referee no. 3), we, like other senior scientists in this field, are unable to reactivate viruses generated using standard BAC protocols.

Since 2011 there have been two significant publications relating to generation of new lytic KSHV BAC systems (see reference 1-2 below). Both proposed exciting potential for KSHV research with which to analyse the contribution of lytically expressed proteins, such as KSHV-TK analysed in our study. Since their publication there has been zero published studies using these systems.

To overcome this limitation we generated an alternative virus, MuHV-4-[g-KSHV-TK], a related gamma-herpesvirus, in which the expression of KSHV-TK is driven under the control of the MuHV-4-TK promoter during lytic replication. Although we understand the limitations of this system we feel given the current technologies it provides an excellent approach with which to analyse the impact of expression of KSHV-TK during lytic replication in the context of a true

gammaherpesvirus infection.
We would be happy to generate recombinant MuHV-4 expressing the mutants of KSHV-TK (KINASE-DEAD, Y65/85/120F mutants) to directly compare the findings obtained when expressing WT KSHV-TK using this replacement viral lytic system.

2. Inducing lytic replication of KSHV in the presence or absence of drugs (Y27632 or Blebbistatin) or Paxillin knockdown, and measurement of resultant virus release.

These experiments would have to be completed using a stable KSHV cell line, for example rKSHV.219, which can be induced to undergo lytic replication using phorbol esters or using a plasmid expressing ORF50. These experiments could not be completed using cells harboring KSHV BAC since they demonstrate inadequate/almost undetectbale lytic reactivation.

The inclusion of drugs or Paxillin knockdown studies would only enable broader generalized statements in lytically replicating cells (using rKSHV.219) with regard to their impact upon either viral release or altered cellular morphology. As expected lytically replicating KSHV (using rKSHV.219) will express a plethora of viral proteins and therefore equating a specific effect relating to the phosphorylation status of KSHV-TK may be hard if not impossible to address.

We have obtained initial data to demonstrate that inclusion of Y27632 during lytic replication of KSHV, using the rKSHV.219 cell line, limits the contraction of lytically replicating cells. In the presence of Y27632 the lytic replicating cells have a more flattened morphology similar to non-induced cell lines. Additionally, we have identical results when Y27632 is added to cells infected with and lytically replicating recombinant MuHV-4 expressing GFP-KSHV-TK (the recombinant virus used in our manuscript- MuHV-4[g-KSHV-TK]).

KSHV-TK potentially has two enzymatic functions, as highlighted by referee number 2, one being our newly identified tyrosine kinase activity and a previously described inefficient thymidine kinase activity. While this weak thymidine kinase activity maybe relevant in some in vivo situations I think that our data clearly demonstrate that KSHV-TK is a potent kinases that must surely exert an influence on the cells the virus is infecting.

We are now considering other journals in which to publish our findings. However, we wondered whether even with its limitations, if we were able to provide the data described above to globally address the contribution of KSHV-TK during lytic replication in relation to the presence of specific drugs, as stated by referee three, whether you would re-consider your decision? Dr. Michael Way will be at the EMBO members meeting later this week and he would be happy to speak with you more directly.

Once again in advance thank you for your time and consideration.

Cited references-

1. Construction of a lytically replicating Kaposi's sarcoma-associated herpesvirus. J Virol. 2011 Oct;85(19):10415-20. doi: 10.1128/JVI.05071-11. Epub 2011 Jul 27.

2. 2012. Construction and manipulation of a new Kaposi's sarcoma-associated herpesvirus bacterial artificial chromosome clone. J Virol. 2012 Sep;86(18):9708-20. doi: 10.1128/JVI.01019-12. Epub 2012 Jun 27.

3rd Editorial Decision 08 November 2013

Thank you for your patience while your arguments were being evaluated by former referee #3. I have now received his/her comments and I am glad to say that they are rather positive.

As you will see below, s/he now believes that a novel manuscript revised along the lines you propose in your rebuttal letter may be suitable for publication in The EMBO Journal. Naturally, once we receive this revised manuscript we might have to contact at least one or two of the former referees before it can be publshed -referee #2, for example, regarding the kinase activity assays s/he proposed- but I would like to invite you to re-submit your study.

My apologies again for the delay in responding. I look forward to seeing your revised manuscript.

Please do not hesitate to contact me if you have any further questions or need further input.

REFEREE REPORT

Referee #3 comments

I believe that the manuscript is improved and they have better described there findings in the framework of KSHV infection rather than the framework of how the known herpesvirus drugs might work. I agree with there statements that if drug inhibition of the alternate TK activities led to inhibition of viral replication or the inhibition of contraction that they claim they have in hand, it is not necessarily ascribable to the TK but it would give the potential that this could be important to viral infection while the negative would indicate that this may not be critical.

As long as the conclusions are appropriately tempered, it could provide potential relevance to their ideas without making the specific BAC recombinant that they describe in their rebuttal that would be a long-term undertaking. In summary I believe that this manuscript would be acceptable at EMBO journal.

Additional Author Correspondence 08 November 2013

This is great news. Many thanks for taking the time to once again consider our manuscript. We will now generate the additional data suggested in our rebuttal letter and re-submit our manuscript as soon as possible.

Many thanks for continued help and positive input.

1st Revision - authors' response 06 February 2014

Referee #1 (Remarks to the Author):

In their manuscript entitled "KSHV thymidine kinase, a tyrosine kinase that disrupts focal adhesions and induces cell contraction", Gill et al characterize the atypical thymidine kinase from KSHV, revealing it to be an autophosphorylating protein kinase that promotes cell contraction through a ROCK-RhoA mechanism. As part of its activity, it induces a dislocation of FAK and paxillin from focal adhesions. Although several key pieces of information are still lacking (to what does KSHV-TK bind, what are it's kinase targets in addition to itself) this study defines a novel function for a TK enzyme that could contribute to cancer-like behaviour in infected cells.

In general, the experiments are well done. However, at times they suffer from a lack of quantification and some important control experiments are lacking. My specific comments are below, which are intended to address these concerns and make this article suitable for publication.

1. Fig 2A: The authors claim that GFP-KSHV-TK overlaps with a subset of actin filaments, but this is difficult to appreciate without an overlay for comparison.

We have now included an additional set of images showing individual actin and GFP-KSHV-TK channels as well as a merged image. These images clearly show that GFP-KSHV-TK filaments aligning with actin stress fibres.

2. An important point that the authors mention is that cells increase their stress fibres before they start to contract: there is no qualitative or quantitative example of this. A movie of Lifeact-expressing cells would be useful to appreciate the cytoskeletal changes that occur prior to contraction, rounding and blebbing.

We have now provided the requested quantification at 16h post transfection in Figure S2A since during this time frame the cells undergo the mots pronounced changes in morphology. This quantification clearly shows that GFP-KSHV-TK but not GFP alone induces actin stress fibres prior to cell rounding.

3. Fig 3: this figure suffers from a lack of quantification. Some quantitative method should be used (for example flourescence units per unit area compared to GFP controls) to determine by how much phosphorylation and/or localization is reduced in KHSV-TK-expressing cells.

We have now quantified the total cell intensity for phospho-paxillin in GFP and GFP-KSHV-TK expressing cells as requested. Representative images are shown in Figure S3B and the drop in fluorescence intensity has been added to the main text on page 7.

4. Treatment with blebbistatin or Y-27632 prevents cell contraction (fig 2); does it have any effect on the phosphorylation status of FAK and paxillin in FAs?

Treating cells with either drug in the absence of KSHV-TK does not alter the staining of phoshotyrosine, phospho-Paxillin Y31 or phospho-FAKY925 at focal adhesions. We have included an example of these data in Figure S2B.

5. Fig 4D: it is impossible to tell how FAKY925 is affected in this panel since the cells shown in Fig 4D and Fig 3A look quite different from one another. Staining is very bright in 4D.

We agree that comparing the effects of KSHV-TK on focal adhesions in Fig. 3A (transfected GFP-KSHV-TK) and 4D (virally expressed GFP-KSHV-TK) is not so clear. It is also not a like for like experiment given the different expression methods. We have now replaced panel D and E in figure 4 in our original manuscript with a single panel (now panel D) that compares the staining for focal adhesion proteins in cells infected with the parental MuHV-4 or the recombinant MuHV-4 expressing GFP-KSHV-TK. We feel this comparison of the effects of the presence or absence of GFP-KSHV-TK in MuHV-4 infected cells makes it easier to see the direct impact of GFP-KSHV-TK on focal adhesion integrity.

6. Fig 7B: TK-DEAD does not come down in FAK pulldowns. The most likely explanation for this is that the autophosphorylation sites provide binding sites for KSHV-TK to form a complex that includes FAK. Therefore, my question: can the Y65/85F mutant be pulled down with FAK? Connected to this, the sentence in the discussion, "The ability of KSHV-TK to associate with FAK only occurs when the kinase is auto-phosphorylated on tyrosines 65 and 85," has not been shown here.

We have now included an IP experiment and show that the Y65/85F and Y65/85/120F mutants maintain their association with FAK (show in Figure 7A-B). We have also stated in the discussion that the although both the Tyrosine 65 and 85 are required to induce the focal adhesion disassembly observed when expressing the WT protein there must be an additional protein(s) that link TK through FAK to intracellular signalling pathways (Discussion, Page 14, Paragraph 1). Consistent with the data presented in our original submission, in contrast to both WT and Tyrosine mutants of KSHV-TK (Y65/85F and Y65/85/120F) that retain their ability to associated with FAK, the KINASE-DEAD mutant does not show any association with FAK. Therefore we can show that KSHV-TK only interacts with FAK when it retains its kinase activity.

Referee #2 (Remarks to the Author):

In this paper the authors have reinvestigated the functions of the KSHV virally-encoded thymidine kinase in inducing cellular phenotypes. They started by showing that expression of KSHV-TK in HeLa cells induced cell contraction and blebbing, and used RhoN19 and Y27632/blebbistatin to show that this was dependent on Rho and ROCK-mediated actomyosin contraction. Consistent with this, GFP-KSHV-TK expressing cells exhibited elevated levels of RhoA.GTP. They went on to demonstrate that GFP-KSHV-TK expression disrupted the integrity of focal adhesions, causing decreases in total P.Tyr, and FAK pY925/paxillin pY31 levels in focal adhesions, as well as reducing the levels of FAK, paxillin and zyxin proteins. Next, they replaced the endogenous TK gene in MuHV-4 herpesvirus with GFP-KSHV-TK by recombination. Infection with MuHV-4[g-KSHV-TK] virus but not MuHV-4 itself caused disruption of focal adhesions. Interestingly, they found that GFP-KSHV-TK was phosphorylated on tyrosine in MuHV-4[g-KSHV-TK] infected cells, and that a TK-dead mutant GFP-KSHV-TK had greatly reduced levels of P.Tyr, and also failed to induce cellular morphology changes and decrease pY925 FAK and pY31 paxillin levels. Tyr65 andTyr85 were identified as GFP-KSHV-TK Tyr phosphorylation through an N-terminal deletion series and individual Tyr to Phe point mutations. The Y65/85F GFP-KSHV-TK mutant failed to induced morphological changes, and, like GFP-KSHV-TK-DEAD, GFP-KSHV-TK, Y65/85F failed to increase Rho.GTP levels. Finally, they showed that GFP-KSHV-TK expression reduced the association of FAK with paxillin but did not affect FAK association with c-Src. Moreover, WT but not TK-DEAD GFP-KSHV-TK coprecipitated with FAK, and siRNA-mediated depletion of paxillin was found to decrease the ability of GFP-KSHV-TK to cause morphological changes or loss of proteins from focal adhesions.

The conclusion that KSHV-TK has an autophosphorylating activity that leads to phosphorylation of two Tyr in its N-terminal region, and that these phosphorylated Tyr cause dramatic cytoskeletal and focal adhesion morphology changes in infected cells is intriguing, and suggests that KSHV TK may have two distinct functions in the infected cell. However, this is the first time that GFP-KSHV-TK has been reported to be phosphorylated on Tyr, and more rigorous evidence is required to establish this unequivocally. For instance, the authors need to do additional experiments to demonstrate that the anti-P.Tyr MAb PY99 signal is really due to P.Tyr, e.g. treatment with recombinant PTP. Moreover, the sites of Tyr phosphorylation were identified only indirectly by mutations, and some direct evidence that Tyr65 and Tyr85 are phosphorylated would be reassuring (e.g. MS data or sequence-specific antibodies to P.Tyr65 or P.Tyr85). In addition, the fact that mutation of these two Tyr to PHe abolishes the ability of KSHV to induce morphology changes does not strictly mean that this is due to a lack of phosphorylation of these residues, because there could simply be a requirement for the OH groups on these two Tyr.

The other main weakness is that there are no true mechanistic insights into how these two Tyr are autophosphorylated, and there are a number of questions in this regard: does autophosphorylation occur cis or in trans; can the isolated catalytic domain phosphorylate the N-terminal fragment in trans or any other substrate protein; and, are these two Tyr the only residues phosphorylated in KSHV TK - Ser and Thr residues could also be phosphorylated- an MS/MS analysis of the isolated KSHV TK protein would be revealing. Most importantly, in order to establish that these Tyr are autophosphorylated by an activity intrinsic to KSHV TK rather than being phosphorylated by another tyrosine kinase in the cell (this is a real possibility given the association of

KSHV with FAK/Src - see point 6) in vitro phosphorylation experiments with purified bacterially-expressed WT and DEAD KSHV TK protein are essential. This would also allow the authors to test if KSHV-TK can phosphorylate other proteins and with what specificity.

The reviewer has raised a number of very important points that we have done our best to address.

1. We have shown that in contrast to the TK's from the gammaherpesviruses EBV and MuHV-4, the KSHV-TK is autophosphorylated when expressed in E. coli. Moreover, this activity is dependent on its kinase domain and not an E. coli kinase, as the Kinase dead GST-KSHV-TK mutant is not phosphorylated.

2. We have performed mass spec analysis of GST-KSHV-TK purified from E. coli. This analysis confirms that tyrosine 65 and 85 are phosphorylated by the KSHV-TK kinase domain (see Fig. S8). In addition, this analysis has also shown that tyrosine 120 is also phosphorylated. We have looked at the impact of the triple tyr mutant on Rho activation, ability to associate with FAK/Paxillin, focal adhesion integrity and cell morphology.

3. We have examined whether the C-terminal kinase domain can phosphorylate the N-terminal half of KSHV-TK when co-expressed in cells. The data in Fig. 5G shows that phosphorylation only occurs in the intact full length protein and not in trans.

The other major unanswered question is how P.Tyr65/P.Tyr85 are able to induce the observed cytoskeletal changes; do they bind to SH2/PTB domain proteins or are they acting in some other way; how does KSHV TK associate with FAK (what FAK domain is involved, and can the phosphorylated N-terminal fragment of KSHV-TK or full-length KSHV-TK bind FAK in vitro?), and how does KSHV-TK displace paxillin from binding to the FAK FAT domain; and, why does MuHV-4 TK not causes these effects if it can also be phosphorylated on Tyr?

MuHV-4-TK and KSHV-TK do show sequence conservation around tyrosine residues 65 and 85. Tyrosine residue 120 in KSHV-TK shows limited sequence conservation in MuHV-4-TK. However, we have no idea which tyrosine residue(s) are phosphorylated in MuHV-4-TK. The kinase domain of MuHV-4-TK is clearly not a tyrosine kinase. Consistent with this, the tyrosine phopshorylation of MuHV-4-TK is dependent on Src family kinases as it is inhibited by PP2 and lacks phosphorylation when expressed in a Src/Fyn/Yes deficient cell line ($SYF^{-/-}$). The phosphorylation of MuHV-4-TK is also rescued when it is co-expressed with SRCGFP in the $SYF^{-/-}$ (MBG unpublished observations/manuscript in preparation). We are happy to provide this supportive data to the referees if required for confirmation. This does not rule out that the equivalent tyrosines in MuHV-4-TK are not phosphorylated albeit by host Src family kinases. However, our pull down data with KSHV-TK and its kinase dead and triple tyr mutants clearly shows that its association with FAK/Paxillin is dependent on its tyrosine kinase activity and not its autophosphorylation. MuHV-4-TK expression has no impact upon focal adhesion integrity and cell morphology, in contrast to that observed when expressing KSHV-TK.

Moreover, our data clearly show that KSHV-TK mediated disruption of focal adhesion integrity and induction of cell rounding is dependent on both its autophosphorylation and its kinase domain, based on the absence of a phenotype with the kinase dead and triple tyrosine mutants. These two mutants, however, have different impacts on the association of KSHV-TK with FAK and paxillin. The most straightforward interpretation is that disruption of focal adhesion integrity and induction of cell rounding is dependent on autophosphorylation of KSHV-TK and phosphorylation of addition cellular targets. Identification of these additional targets will undoubtedly be important to understand how KSHV-TK exerts its effects. However, at this stage we feel that it is beyond the scope of the current study, which has already provided important new insights into the activity of KSHV-TK, namely it is a tyrosine kinase.

We have not further analysed the specific domain interaction between FAK and KSHV-TK in this study since both the double and triple tyrosine mutants of KSHVTK retain their ability to interact with FAK. Therefore we recognise that an additional protein(s) is required to promote KSHV-TK induce focal adhesion disassembly beyond the requirement of FAK. The identification and characterisation of KSHV-TK interacting proteins is part of our ongoing and mechanistic studies to determine precisely how KSHV-TK impacts upon cell signalling pathways.

Other points: 1. Figure 2: There was significant cell-to-cell variability in the level of expression of GFP-KSHV-TK, and it is not clear that the cell morphology and the actin cytoskeletal phenotypes actually correlated very well with the levels of GFP-KSHV-TK expression.

The level of expression of GFP-KSHV-TK does vary from cell to cell. A similar variation is also seen in GFP alone, which is not unexpected in transient expression experiments. However, there does not appear to be a correlation between expression level and phenotype as even low levels of GFP-KSHV-TK still induce cell rounding, presumably as it is a potent tyrosine kinase that is not regulated/inhibited by the cell.

We have now also provided quantification of changes in actin stress fibres and cell rounding in cells expressing GFP and GFP-KSHV-TK in Figure S2A. We hope this data addresses the reviewers concerns.

2. Figure 3: The GFP-KSHV-TK-expressing cells of interest need to be labeled with arrowheads in the GFP panel as well as in the protein staining panel of the same cells. Can decreases in pY925 FAK and pY31 paxillin be observed by immunoblotting whole cell proteins, or by immunoblotting FAK and paxillin immunoprecipitates? Is there a change in the total level of paxillin and zyxin upon KSHV-TK expression (this is not clear from Figure 7A)?

We have added arrowheads as requested. We have tried to look at the levels of pY925 FAK and pY31 paxillin by immunoblot. In our hands the antibodies have not worked well or consistently for immunoblotting. Given our transfection efficiencies are not 100% we have quantified the levels of pY925 FAK and pY31 paxillin at focal adhesions (see reviewer 1). This analysis reveals that the levels of phosphorylation of FAK and paxillin are reduced in GFP-KSHV-TK expressing

cells (Fig. S3). To further address this reduction by immunoblot analysis we have immunoprecipitated FAK and Paxillin in both GFP and GFP-KSHV-TK expressing cells and blotted the precipitates for pan phosphotyrosine. A reduction in both the tyrosine phosphorylation of FAK and Paxillin in KSHV-TK expressing cells can observed as shown in Figure S3B.

3. Figure 4B: It is unclear from the legend and the text exactly what was done here, and MuHV-4 Revert is not properly described, forcing the reader to look up Coleman et al. (2003) to find out what it is. One assumes that these are immunoblots of whole cell proteins with PY99 and anti-GFP. If these are whole cell protein blots, then one cannot be certain that the P.Tyr-positive band in the MuHV-4[g-KSHV-TK] lane is in fact GFP-KSHV-TK, and the authors need to isolate the GFP-KSHV-TK protein by immunoprecipitating with anti-GFP antibodies and then blot this with anti-P.Tyr antibodies, using a PTP-treated sample as a control. Is the band in the MuHV-4 and MUHV-4 Revert lanes P.Tyr-containing MuHV-4 TK? If this the case, why does MuHV-4 TK not induce changes in cell morphology? This issue is not discussed, and raises the question of what is special about KSHV-TK compared to MuHV-4 TK. Based on a cursory sequence comparison of the KSHV and MuHV-4 TKs, the two Tyr appear to be conserved, and the sequences around "Y65" are quite similar. Finally, the whole gel needs to be shown to demonstrate how the global P.Tyr pattern in the infected cells was changed (this would also need an uninfected cell control).

We have modified Figure 4 for further clarity. We have removed the data for the Revertant and TK- viruses since they did not add anything to the main message of the paper. The recombinant MuHV-4 virus expressing GFP-KSHV-TK was generated using standard BAC mutagenesis as detailed in the materials and methods. We now show cells infected with WT versus GFP-KSHV-TK expressing recombinant virus to allow a direct comparison to be made (Fig. 4C). We have also included complete blots to show all changes to the global levels of phosphotyrosine (Fig. 4B) as requested. It is clear that the predominant proteins modified by tyrosine phosphorylation are the viral encoded TKs. The phosphotyrosine band for GFP-KSHV-TK corresponds to the GFP-positive signal. The identity of the GFP-positive protein was confirmed as GFP-KSHV-TK by mass spec analysis of a anti-GFP immuno-precipitation. This data has not been included in the paper but can be provided if the reviewer feels it is necessary.

The fact that MuHV-4-TK does not induce changes in focal adhesion integrity and cell rounding has already been discussed above in relation to an earlier question raised by the reviewer.

4. Figures 4 and 5: Can a dissociation-of-function catalytic domain mutant be made that lacks autophosphorylating activity but retains TK activity (and vice versa)?

This is a very interesting idea. However, at this point it will be difficult to address since KSHV-TK has almost undetectable Thymidine kinase activity when assayed using conventional TK assays (Gustafson EA et al., J Virol. 2000, 74: 684–692.). We also feel it will be easier to address once we have some structural data.

5. Figure 6: Based on these data, it appears that Y65F and Y85F KSHV-TK are both able to induce morphology changes, whereas the Y65/85F mutant cannot. This implies

that pY65 and pY85 have redundant functions even though the sequence around them is very different.

From our additional studies we have also identified an additional tyrosine residue at amino acid residue 120 that is auto-phosphoyrlated in KSHV-TK. Unlike the expressed WT protein that induces disassembly of focal adhesions the expression of the double (Y65/85) and triple (Y65/85/120F) tyrosine mutants fail to induce cellular remodelling. With the triple mutant showing a phenotype almost identical to the mock transfected cells- flattened morphology and normal assembly of focal adhesions. We have also added extra data to demonstrate that when expressed during lytic replication in the context of a recombinant MuHV-4 virus, the expression of the KSHV-TK triple mutant (Y65/85/120F) does not induce cell rounding (see Figure 6 panel G). The latter results are consistent with those obtained in single gene expression studies. It is feasible that in the context of the full protein the three tyrosine are used to interact with a SH2 domains of neighbouring proteins (or one protein with two tandem SH2 domains). It would be interesting to examine what cellular protein(s) bind the individual phosphorylated tyrosines but at this stage we feel that's one for the future and beyond the papers scope.

6. Figure 7: The authors have deduced that KSHV-TK is autophosphorylated in vivo, because the KSHV-TK-DEAD mutant shows reduced P.Tyr content. However, since GFP-KSHV-TK-DEAD does not associate with FAK/Src, it is possible that GFP-KSHV-TK is in fact not autophosphorylated but rather is phosphorylated in trans by FAK or Src. Does the association of KSHV with FAK require phosphorylation of Tyr65/85 - the authors need to test this with the F65/85 GFP-KSHV-TK mutant. Does MuHV-4 TK associate with FAK?

KSHV-TK can be expressed in FAK- and Src- (Yes and Fyn) deficient SYF cells and is still tyrosine phosphorylated. More importantly, KSHV-TK expressed and purified from *E. coli* is phosphorylated on three tyrosine residues (see Figure 5F for immunoblots and Figure S8 for MS/MS analysis). This is in contrast to MuHV-4 TK which we now know is phosphorylated by cellular enzymes (Src/Fyn and Yes). The tyrosine phopshorylation of MuHV-4-TK is dependent on Src family kinases as it is inhibited by PP2 and lacks phosphorylation when expressed in a Src/Fyn/Yes deficient cell line ($SYF^{-/-}$). The phosphorylation of MuHV-4-TK is also rescued when it is co-expressed with SRC-GFP in the $SYF^{-/-}$ (MBG unpublished observations/manuscript in preparation). We are happy to provide this supportive data to the referees if required for confirmation.

7. Figure 7D and E: The authors state that depletion of paxillin did not affect Tyr phosphorylation of KSHV TK but do not show this directly. It is somewhat surprising that depletion of paxillin per se did not have a more dramatic effect on focal adhesion morphology.

Paxillin depletion increases Rac1 activity (*) and will maintain the cell in a more ruffled and flattened morphology. Therefore the expression of KSHV-TK a protein that normal induces cell rounding will be counteracted by the depletion of Paxillin.
(*) Nicholas O. Deakin and Christopher E. Turner. **Distinct roles for paxillin and Hic-5 in regulating breast cancer cell morphology, invasion, and metastasis.** Mol Biol Cell. 2011. 22(3): 327–341.

Referee #3 (Remarks to the Author):

This group has previously shown that KSHV TK does not have strong TK activity and induces cell shape changes. In this manuscript they begin to study the cell shape changes induced by KSHV TK. They find that KSHV-TK induces cell contraction dependent on ROCK and RhoA and there is a decreased expression of surface focal adhesions along with other changes associated with focal adhesions like paxillin. They also find that tyrosine phosphorylation of KSHV-TK is necessary for these effects. They replace the TK of MuHV-4 with the TK of KSHV and find that during lytic infection in cell culture they see similar effects as they saw with the KSHV-TK alone but they do not see these effects with the natural MuHV-4 TK. While the phenotypic effects of the KSHV-TK are interesting and provide evidence for functions other than a thymidine kinase for the KSHV protein, there is little discussion of the role of this effect in KSHV biology or lytic replication and there is no evidence that this might be important for KSHV replication or any part of KSHV biology. Also, they spend a great deal of the introduction and discussion stating that for understanding how antivirals that work against TK work we need to understand all the functions of TK. While the other functions are definitely of interest, only an understanding of the thymidine kinase activity is needed, other functions play other roles and are unrelated to the antivirals. There are also some places in the manuscript that need additional data to clearly make their points. These are listed below.

1. Overall, the effects found are an interesting phenomenon but the actual outcome of these effects for the virus are not discussed. There is no evidence that KSHV lytic replication leads to these effects. While they state there is no lytic system for KSHV, there are ways to induced KSHV lytic replication in a sub-set of cells, and through their microscopy, they could identify these and determine if there is a change in the focal adhesions or paxillin etc... This might provide some meaning to the phenomenon they describe.

To be more specific, the authors should induce lytic replication in infected cells with Rta (expressed from a transfected plasmid or adenovirus as has been described in the literature) and the block the downstream changes induced by KSHV TK using the drugs described or the siRNA to paxillin. They could then quantify the virus released and thereby determine if the KSHV-TK plays a critical role in lytic replication of KSHV. This would provide relevance of this interesting phenomenon they describe.

We have now included an extra supplemental Figure (Figure S6) showing that KSHV infected cells undergo rounding during lytic replication. The observed cell rounding can also be blocked by the addition of the ROCK inhibitor, Y-27632. Although these findings are analogous to that observed when expressing the KSHV lytic protein KSHV-TK, the results obtained using whole virus relate to KSHV lytic replication per se. Further detailed studies to precisely define the exact contribution of KSHV-TK and its phosphorylation to this observed phenotype is part of our ongoing studies, although limited at present due to the absence of a robust KSHV BAC system with which to analyse the impact of individual viral protein during lytic replication. Additionally, we also have provided data to demonstrate the cell rounding induced by the expression of KSHV-TK when cells are infected with the recombinant MuHV-4-gKSHV-TK virus can be blocked by the inclusion of Y-27632 (Figure S6_panel A).

2. In figure 2 they describe co-localization of KSHV-TK with Actin stress fibers. This is very difficult to see without an overlay picture with different colors as the TK is all over the cell and the Actin fibers are widely distributed.

We have included a merged image here to show how the TK and actin can run parallel to each other.

3. In figure 3 they describe the disassembly and loss of phosphorylation of focal adhesions and FAK. They would enhance this loss with a more quantitative western blot analysis of phosph-FAK.

We have included an extra panel in Supplemental data to show the corrected total cell fluorescence to demonstrate the reduction in phospho-Paxillin Y31- demonstrating the decrease in total cell fluorescence intensity in cells expressing KSHV-TK when compared to those expressing GFP (Figure S3B).

4. In figure 4D it looks like p-FAK is increased all over the cytoplasm in the presence of KSHV-TK. What is this localization and increase in phsopho-FAK?

Phospho-FAK appears to be more cytoplasmic in KSHV-TK expressing cells. Since the both proteins associate the TK might be sequestering this focal adhesion protein in the cytoplasm and preventing its trafficking or recruitment to focal adhesions.

5. What is the logic that to understand the ability of antivirals to work against KSHV, all the functions of TK must be known. Knowing that the KSHV TK does not have strong thymidine kinase activity leads to the understanding of why some antivirals don't work. While the other functions may be interesting, they do not shed light on the kinase activity per se.

If the KSHV-TK can act as a tyrosine kinase then this is a powerful way for the virus and one that is separate from its function as a TK to modulate the normal function of the cell. Cells tightly regulate tyrosine phosphorylation of proteins as this modification can dramatically affect the activity of a protein leading to changes in a multitude of cellular processes including cell signalling and migration. Indeed, aberrant activation or mutation of Src/Abl and FAK kinases is a hallmark of cell transformation and tumour development and invasion. Therefore in a quiescent "normal" cell the presence of phosphorylated KSHV-TK as well as its ability to tyrosine phosphorylate host proteins is likely to lead to the activation and/or inhibition of multiple signalling pathways regulating essential cellular processes such as adhesion and migration. Thus it is important to understand the tyrosine kinase activity of KSHV-TK to help faciliate identifying drugs that will inhibit its activity, as they may be useful to treat diseases associated with lytically replicating KSHV.

4th Editorial Decision 14 March 2014

Thank you again for the re-submission of your manuscript on KSHV TK and your patience while it has been evaluated. I apologize for the delay in responding, but we have only now received the comments from the referees. Your manuscript has been evaluated by former referees #1 and #3 who, although positive in general, still point out to a number of concerns that unfortunately preclude de publication of your study in The EMBO Journal.

In essence, while both referee agree on the high potential interest and novelty of the conclusions presented, they also consider, especially referee #1, that your study is not sufficiently developed for The EMBO Journal, basically but not exclusively due to a somewhat reduced mechanistic insight. That being said, I took the liberty to discuss your study with Nonia Pariente, one of the editors at our sister journal EMBO Reports. As you may know, they publish short (5 figures maximum), single message papers reporting conceptually novel findings, and emphasize functional relevance over detailed mechanistic insight, which we require at The EMBO Journal. I am glad to say that they would be willing to accept a suitably revised version of your manuscript, provided the following points are addressed:

- strengthen the data supporting that KSHV-TK has an intrinsic tyrosine autophosphorylation activity by performing the in vitro biochemistry that referee 1 requests in his/her point 2.

- discuss the issues raised in referee 1's point 3 and how they envision that a lytic cycle protein could have a role in oncogenesis, as mentioned by referee 3 in point 2.

- in order to bolster the relevance of the reported cell morphology changes in KSHV life cycle, we would strongly suggest the authors quantify the virus released after induction of lytic replication with Rta in the presence or absence of ROCK inhibitor, which they have shown blocks the changes in cell morphology, as was originally requested by referee 3.

Please be aware that, should you decide to transfer your manuscript to EMBO reports, it will most likely need shortening or reorganization prior to publication. EMBO reports editors will aid in this process if necessary.

I am sorry I can not be more positive this time but I hope you will view the possibility of a transfer favorably. If this is the case, please use the link below to transfer the manuscript directly.

REFEREE REPORTS:

Referee #1:

EMBOJ-2013-87154R1 Gill

In the resubmitted version of this paper, the authors have obtained direct MS evidence that the N-terminal domain of KSHV-TK is phosphorylated at three N-terminal Tyr, and that this phosphorylation requires the N-terminal domain to be physically attached to the catalytic domain. They had previously identified Tyr65 and Tyr85 through mutation, and here they confirmed Tyr65/85 phosphorylation by MS, and added Tyr120 as a new site. They showed that a triple Y65/85/120 KSHV-TK mutant is not phosphorylated on Tyr, and fails to induce cell contraction and changes in focal adhesions, and based on the effects of mutating these sites individually and in combination, they conclude that autophosphorylation of all three sites is important for morphological effects of KSHV-TK expression.

However, a number of issues remain:

1. Mechanistically, it is unclear exactly how these N-terminal Tyr are phosphorylated - does this occur in cis (if so, is it physically possible for the N-terminal region to reach around the C-terminal

catalytic domain to present Tyr120 to the active site for phosphorylation?), or does phosphorylation perhaps occur in trans within a KSHV-TK dimer. The authors should note that their use of GST-KSHV-TK fusion proteins, which are themselves dimers, may have facilitated transphosphorylation. What happens if KSHV-TK is expressed as a monomer in E. coli, e.g. as a His-tagged protein? The new data in Figure 5G indicate that the N-terminal domain of KSHV-TK was not phosphorylated on Tyr when expressed together with the catalytic domain in mammalian cells, but it is not clear that the catalytic domain fragment was active, since the GFP moiety at its N-terminus may interfere.

2. The authors have relied on the finding that WT but not kinase-dead KSHV-TK isolated from bacteria is phosphorylated on three N-terminal Tyr residues to conclude that this is an intrinsic activity, but they would strengthen this conclusion with some biochemistry using purified recombinant KSHV-TK purified from bacteria to show it can re-autophosphorylate in vitro after prior dephosphorylation with lambda phosphatase. This approach would also enable the authors to test whether dThd blocks this activity (i.e. do protein Tyr and dThd compete for binding to the same site in the catalytic domain?), and also whether the N-terminal domain can be phosphorylated in trans by full length KHSV-TK.

3. Did the authors check for Ser/Thr phosphorylation of the N-terminal domain of KSHV-TK (based on the abbreviated description of the of h MS analysis it is not clear whether they focused only on identifying possible pTyr sites)? What happens if the Tyr residues are mutated to Ser or Thr? How many other Tyr are there in the N-terminal domain sequence?

4. How does the unphosphorylatable mutant KSHV-TK bind to FAK, whereas the kinase dead mutant does not - perhaps the kinase-dead mutant is misfolded because it cannot bind ATP (it might be better to use a D362A kinase-dead mutant)(the FAK FERM domain might bind to the pTyr residues in KSHV-TK but they are not needed) for FAK association). Since FAK is needed for the morphological effects induced by KSHV, it would be interesting to test whether the level of FAK pY397 is increased and whether FAK and Src inhibitors also block KSHV-TK-induced cell contraction.

5. In the end, it remains unclear how phosphorylation of these three Tyr results in disruption of focal adhesion structure and signaling. They have not identified potential SH2 domain binders for the KSHV-TK pTyr residues, which might give clues, and there is no discussion of this possibility. Based on sequence, one would predict that pY65 and pY85 might bind ABL and PLCg among other SH2 proteins, and that pY120 might bind p85/PI-3K among other SH2 proteins. If pY120 can bind and activate PI-3K, this could lead to RhoA activation, and it would be therefore informative to analyze a Y120F single mutant KSHV-TK to see whether this diminishes RhoA activation and also whether a PI-3 kinase inhibitor might moderate the morphological effects caused by KSHV infection.

Points: 1. The double asterisks in Figure 4B need to be defined.

2. Figure 5: Panel G is not described in the legend (panel F is also not adequately described). In addition, the residue numbers for the N-terminal fragment expressed as a GFP fusion in mammalian cells (what cells - HeLa?) need to be added. From looking at panel G, it is hard to figure out what was done - there appears to be a single lower GFP-positive band of identical size in the three right hand lanes. Is this the GFP-N-terminal fusion protein? If so, why is this band in the CT alone lane?

3. Figure 6A: The D1-100 KSHV-TK was not Tyr phosphorylated (presumably this analysis was done in E. coli, but this is not stated in the legend). How did the authors find pTyr120 in their MS analysis? It would be worth testing a Y120F single mutant, since this pTyr would be expected to bind a different set of SH2 proteins, whereas pTyr65 and pTyr85 might have redundant SH2 binding properties.

4. Figure 6C-E: Expression of the Y65/85/120F mutant does seem to cause some perturbation of cell morphology and focal adhesion distribution, but nothing like as dramatically as WT KSHV-TK does.

Referee #3:

This group has previously shown that KSHV TK does not have strong TK activity and induces cell shape changes. In this revised manuscript they begin to study the cell shape changes induced by KSHV TK. They find that KSHV-TK induces cell contraction dependent on ROCK and RhoA and there is a decreased expression of surface focal adhesions along with other changes associated with focal adhesions like paxillin. They also find that tyrosine phosphorylation of KSHV-TK is necessary for these effects. They replace the TK of MuHV-4 with the TK of KSHV and find that during lytic infection in cell culture they see similar effects as they saw with the KSHV-TK alone but they do not see these effects with the natural MuHV-4 TK. They now show these effects occur during KSHV lytic induction and while they cannot show that this is due to the KSHV TK it is provoking.
The authors have adequately responded to the reviewers' comments. However, there are a couple places where the authors make statements about KSHV that are not as accurate as they could be.
1. They state that there is no robust Bac system. In the last two years a more robust Bac system has been published (J. Virol. 2012, 86:9708-20) so they should remove this statement.
2. In their discussion of the potential role of these alternative TK functions in KS, they need to better discuss that the role would be in the low percentage of infected cell undergoing lytic replication and are less likely to be directly involved in oncogenesis unless they can show expression during the latent phase.

Appeal 21 March 2014

Many thanks for your feedback regarding our manuscript (EMBOJ-2013-87154R1). We are of course very disappointed with the ultimate outcome given the time and effort we have made to satisfy, not just some but essentially all of the comments raised by the three referees in their initial review.

Although we respect the extensive comments of our referees, we are totally confused by one fundamental statement- "especially referee #1, that your study is not sufficiently developed for The EMBO Journal, basically but not exclusively due to a somewhat reduced mechanistic insight. "

Referee 1

In the initial review process referee 1 did not mention that the lack of mechanistic insight was a factor preventing the publication of our manuscript. In fact referee 1 stated: "In general, the experiments are well done. However, at times they suffer from a lack of quantification and some important control experiments are lacking. My specific comments are below, which are intended to address these concerns and make this article suitable for publication."

Referee 1 raised 6 points of concern to be addressed that we made every effort to complete as thoroughly as possible, which is partly why it took so long to send back the revised MS.

Referee 3

Referee 3 wanted us to include data to show that the drugs that blocked ROCK signalling or siRNA that knocks down Paxillin effected virus output of KSHV infected cells. As we clearly explained in our letter the novel phenotype we were examining in this manuscript related to the unique function

of the thymidine kinase to act primarily as a tyrosine kinase and remodel focal adhesions.

As has been published for a closely related gammaherpesvirus TK (MuHV-4), the TK is not actually required in vitro for virus replication (J Virol. 2003 77:2410-7) but is essential for in vivo pathogenesis (J Gen Virol. 2009 90: 1461-1470). Therefore as we clearly explained in our rebuttal letter, specifically addressing the concerns of referee 3, blocking either TK, in terms of its residual thymidine kinase activity or downstream signalling, would not provide any additional insight into the role relating to the novel tyrosine kinase activity we have documented in our study. After clearly communicating our concerns relating to the proposed experiments cited by Referee 3 in our rebuttal letter, referee 3 responded with the following statement.

"As long as the conclusions are appropriately tempered, it could provide potential relevance to their ideas without making the specific BAC recombinant that they describe in their rebuttal that would be a long-term undertaking. In summary I believe that this manuscript would be acceptable at EMBO journal". (Email correspondence, 8.11.2013).

Actually we did generate a new BAC in which the expression of the mutant KSHV-TK could not induce focal adhesion disassembly in a viral context, consistent with our transfection studies. This was completed to provide stronger support for our initial findings. We also demonstrated that Y-27632, the drug that blocks KSHV-TK induced focal adhesion disassembly in transfection studies, also prevented cell rounding in lytically replicating KSHV cells. These experiments directly addressed the concerns of referee 3.

The only point we did not address was the release of virus from KSHV infected cells in the presence of Y-27632. As stated above, Gammaherpesvirus TK's are not required for in vitro replication of the virus in cell culture and have no impact on viral titers. Given this, quantifying virus release after inhibiting ROCK-Myosin II downstream of KSHV-TK function would yield no meaningful or informative data relating to the unique ability of KSHV-TK to act as a tyrosine kinase and promote the remodelling of focal adhesions and cell morphology.

Given the classical published literature I appreciate the lack of understanding of how the expression of viral lytic proteins could contribute to a viral pathogenesis since lytic replication of herpesviruses is traditionally thought to represent the end phase of infection (apoptosis and virus released). Most cells infected with KSHV undergo tight latent infection and only undergo lytic replication following induction of the lytic cycle. However, from more recent research (doi: 10.1016/j.chom.2013.03.009.) it has now been shown by a world leader in KSHV research that lymphatic endothelial cells infected with KSHV can be continually propagated whilst expressing a wide array of lytic proteins. As the authors quote, (Page 437) "In the KSHV field, there has been significant suspicion that this simple model may not always be applicable. It has been suggested that traditional in vitro models of KSHV infection, in which latency is generally the default pathway, may not reproduce important features of viral gene expression relevant to KS (Mesri et al., 2010). There is good evidence that both latent and lytic gene products may play important roles in KS pathogenesis (Ganem, 2010; Mesri et al., 2010). While the effects of latent gene expression on cell survival and signaling have been much commented upon, there is considerable evidence linking lytic expression to KS development.

Therefore although the expressed KSHV-TK may not contribute anything to virus replication in terms of nucleoside phosphorylation (since its an inefficient thymidine kinase), as we have clearly discussed, its expression and ability to directly modulate cellular signalling pathways and cellular adhesion might significantly contribute to the oncogenic potential of KSHV, a potent human oncogenic virus.

I hope our detailed response will highlight why we are very disheartened by the ultimate outcome of this review process and hope that you might reconsider your decision.

I would like to thank you for discussing our paper with your sister journal, EMBO reports. However, there is still the fundamental issue that the new suggestions by the reviewers will add nothing and/or are not possible to do in addition to the difficulties in fitting into the EMBO reports format with out loss of key information and readability.

5th Editorial Decision 25 March 2014

Thank you very much for your e-mail. I have discussed your case again with the editorial team and I regret to say that we see no option but to maintain our decision.

We appreciate the arguments in your rebuttal letter and we agree with you regarding referee #3, which in any case has a relatively positive opinion on your manuscript. Some concerns remain, such as the ones mentioned in my letter, essentially questioning the physiological relevance of the tyrosine kinase activity for virus pathogenicity or replication. Nevertheless, as explicitly mentioned in my letter, further experimental evidence in this direction is "strongly suggested", but not required for further consideration in EMBO reports.

We also understand your confusion regarding referee #1. Originally, this referee was referee #2, as you can tell according to the concerns expressed initially, which are rather similar to the concerns presented now. A technical issue changed the referee identity (it happens when the number of referees changes from one version to the next) and I forgot to change back these numbers. I apologize for the confusion.

Considering the arguments presented, including the fact that referee #3 is relatively positive but definitely not enthusiastic and that referee #1 (former referee #2) is rather negative, I am afraid that we cannot reconsider you manuscript for publication in The EMBO Journal.

I am sorry again that I cannot be more positive this time, but I hope that new improved version of the manuscript is quickly published somewhere else, possibly EMBO reports if you reconsider their offer.

Thank you very much again for considering The EMBO Journal.

Resubmission 21 October 2014

16/10/2014

We would like to resubmit our revised manuscript entitled, “KSHV-TK is a tyrosine kinase that disrupts focal adhesions and induces Rho-mediated cell contraction” (Manuscript EMBOJ-2013-87154R2-Q). When last reviewed, our paper was rated of high potential interest and novelty. Nevertheless, it was considered that the study was not sufficiently developed for The EMBO Journal as it did not provide sufficient mechanistic insight. It was, however, considered of sufficient standard for EMBO Reports.

In our initial manuscript, we documented the ability of the thymidine kinase expressed by KSHV to act as a tyrosine kinase and autophosphorylate tyrosine residues present within its N-terminal domain. We demonstrated that the expression of this viral kinase promoted the disassembly of focal adhesions and induced cell contraction in a Rho-ROCK-Myosin II dependent manner. We were able to identify three tyrosine residues within its N-terminal domain that are essential for its ability to induce the disassembly of cellular adhesions. We also demonstrated that these phenotypes were also observed in cells lytically replicating KSHV. Although, we acknowledge the limitations of the latter studies given the lack of a robust lytic system for KSHV in which specific genes can be mutated and then analysed using latent versus lytic in vitro replication studies.

Taking on board your concerns as well as those of the reviewers we set out uncover the mechanism by which KSHV-TK induces focal adhesion disassembly. We have now identified the family of oncogenic adaptor proteins, CrkI, CrkII and CrkL, as specific binding partners for two (Y65 and Y85) of the three essential tyrosine residues autophosphorylated in KSHV-TK. This interaction is specific to the SH2 domain of all Crk family members and over expression of the SH2 domain alone is sufficient to block KSHV-TK induced focal adhesion disassembly. It is important to note that Crk related proteins are key cellular adaptor proteins that contribute to the regulation of cellular adhesion. We also demonstrate that both CrkII and CrkL are phosphorylated on their regulatory tyrosine residues (Y221 and Y207 respectively) following binding to KSHV-TK. This phosphorylation state represents an inactivated form for CrkII and CrkL that is known to promote cell detachment from the substrate. Additionally, we have identified the p85 subunit of PI3-K as a binding partner for the third

phosphorylated tyrosine (Y120) in KSHV-TK.

The ability of KSHV-TK to interact with the family of Crk related proteins is of significant interest for two key reasons. Firstly, it provides a mechanistic understanding of how KSHV-TK regulates cell adhesion and contraction dynamics. Second, KSHV-TK is only the second example to date of a viral protein that interacts with the family of Crk family proteins. The contribution of Crk to viral pathogenesis in general remains to be explored and is currently limited to the study of specific strains of Influenza virus.

We feel that the additional data we have provided in our revised manuscript significantly improves our mechanistic understanding of how KSHV-TK promotes focal adhesion disassembly. It also provides an exciting link to explain how viral proteins hijack key cellular oncogenic proteins to modulate cellular dynamics. We hope that you agree that our manuscript is suitable for publication in The EMBO Journal.