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A novel GRK2/HDAC6 interaction modulates cell spreading and motility

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1st Editorial Decision 07 June 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the delay in getting back to you with a decision: this was due to the late return of one of the referee's reports. However, we do now have a complete set of referees' comments, which are enclosed below. As you will see, all three referees find your identification of GRK2 as a regulator of HDAC6 activity to be interesting, but all three also raise a number of significant concerns that would need to be addressed by a major revision of your work.

To summarise the critical points in detail:
- While you clearly show that GRK2 can phosphorylate HDAC6 in vitro, you do not provide sufficiently good evidence that this phosphorylation occurs in cells and is important for HDAC6 activity. This is particularly important since GRK2 can phosphorylate tubulin, which could indirectly impact upon HDAC6-mediated tubulin deacetylation. In this context, mapping the phosphosite for GRK2 on HDAC6 and analysing phosphomutant/phosphomimetic constructs would be critical.
- You focus almost exclusively on the tubulin deacetylation activity of HDAC6, but as the referees point out, cortactin deacetylation may also play a role here, and it would be important to analyse this.
- Further loss of function analysis - ideally using GRK2 knockout cells - to back up your major conclusions would be essential.

Regarding referee 3's comments about statistics, I note that those panels where you do not show statistical significance are those where the data represent only two independent experiments. Ideally, I would encourage you to repeat these experiments again to allow an assessment of statistical

significance, but in case this can not easily be done, I would ask that you show individual data points on the graphs, rather than means. In general, our guidelines are that in cases where n{less than or equal to}3, individual data points are plotted.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

If you have any questions or comments about this revision, please don't hesitate to get in touch.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1:

The manuscript by Lafarga et al. is very interesting. In a previous paper published in EMBO J by the same authors they demonstrate a role of GRK2 for integrin-mediated cell migration. In the current paper they show an additional mode of GRK2 to modulate cell migration and cell spreading. They propose that the catalytic activity of GRK2 promotes HDAC6 activity, which in turn triggers deacetylation of tubulin and finally promotes cell migration and spreading.
Although very interesting, the paper requires attention to three major issues prior to publication:
First, the spreading experiments should be repeated with GRK2-depleted cells.

Second, the authors must demonstrate how the catalytic activity of GRK2 promotes HDAC6 activity, cell migration and cell spreading. GRK2 may function either by directly binding and phosphorylating (?) HDAC6 or indirectly through an adaptor that in turn is inducing the activity of HDAC6. In this respect it is interesting that the authors found that the GRK2-S670A mutant fails to induce HDAC6-mediated de-acetylation of tubulin. A paper by Pitcher et al. (JBC, 1998) provided evidence that GRK2 associates with and phosphorylates tubulin. It could therefore be that GRK2 forms a ternary complex consisting of tubulin/MTs and HDAC6, phosphorylates tubulin/MTs, which then leads to HDAC6 binding to MTs followed by their de-acetylation? It is not clear which protein is phosphorylated by GRK2! Clearly, this aspect of the paper is not well developed.

Third, the role of GRK2-mediated activation of HDAC6 and deacetalytion of tubulin needs to be directly shown in migration/spreading assays. This is particularly important since (a) GRK2 can modulate migration in an HDAC6-independent manner (shown in a previous EMBO J paper by the same authors), and (b) HDAC6 can regulate migration and spreading through additional players such as cortactin (Zhang et al., Mol Cell, 2007). It should therefore be shown whether loss of GRK2 and overexpression of catalytically inactive GRK2 impair de-acetalytion of both, cortactin and tubulin, and to which extent each of them contributes to the cellular defects described in the current

paper. Furthermore, to which extent does the role of GRK2 on GIT/focal adhesion impact cell migration.

Finally, it is unclear to me why the authors use heterozygous GRK2-null cells in their studies. Floxed mice are published and it should be straight forward to derive cells from the floxed mice and subsequently transduce Cre to obtain true GRK2-null cells for this important study.

Specific comments:
Fig. S1A-C: GRK2-depletion of wt MEFs reduces GRK2 levels by about 50% while the functional impairments are similar to the GRK2+/- MEFs with a GRK2 reduction of around 75%. I would have expected more pronounced impairments in siRNA-treated GRK2+/- MEFs. How can this discrepancy be explained? Have the siRNA depletions been rescued with siRNA-resistant GRK2 cDNAs?

Fig. 1C: is the NaB effect on wt cells significant?

Fig. 1D: overexpression of wt GRK2 does not affect tubulin acetylation, while it significantly affects migration in a wound closure assay. The authors argue that localized increases in GRK2 activity may explain these effects. Is it possible that migration represents an activation signal of GRK2 which is missing in resting cells? What could such a signal be?

Fig. 2A-C: the assays do not rule out the possibility that HDAC6 immunoprecipitates GRK2 through its ability to bind tubulin. It has been shown previously that GRK2 can also bind tubulin. Can GRK2 bind HDAC6 in the absence of tubulin? Is HDAC6 binding and phosphorylation by GRK2 a tubulin-dependent or -independent event?

Fig.2F: can the phosphor-acceptor site in DD2 be identified? Does DD2 contain a GRK2 phospho-consensus site? Such an identification and subsequent mutation of the acceptor site would tremendously strengthen the impact of the paper and the observation that the formation of the ternary complex consisting of GRK2/tubulin/HDAC6 is kinase dependent.

Fig. 3D: the GRK2-S670A reduces HDAC6 phosphorylation. Does the pospho-mimicking mutation S760D enhance phosphorylation?

Fig. 4: the images are of poor quality. HDAC6 accumulation at the leading edge is only visible in a single cell of the low magnification image. This is not convincing and has to be improved. In my view HDAC6 levels do not have to be increased at the leading edge. Also an evenly distributed HDAC6 will show an uneven activity if its activators/inactivators are unevenly distributed. Furthermore, the scratch should be indicated with a dotted line.

Referee #2:

Lafarga and colleagues report here a physical interaction between the ubiquitous G protein-coupled receptor kinases member, GRK2, and HDAC6, leading to HDAC6 phosphorylation, which enhances its catalytic activity and extended tubulin deacetylation. The authors propose that this tubulin deacetylation affects microtubule (MT) stability, which in turn modulates cell motility and spreading.

General comments
It seems that the authors have been confused by the widely used HDAC6 qualifier, "tubulin-deacetylase", which somehow shadows the fact that HDAC6 is primarily a protein deacetylase and tubulin is only one of its substrates. This leads the authors to interpret all their findings through the modulation of tubulin acetylation, while some of their observations would better fit with the acetylation of another HDAC6 substrate, cortactin.
The authors seem to be aware of this fact since in the discussion section, the possibility of a connection to other HDAC6 substrates is mentioned at least twice. One example is at the end of the discussion section, where we can read : "...GRK2 by increasing the HDAC activity of HDAC6 towards a-tubulin (AND PERHAPS TOWARD OTHER SUBSTRATES) would favour aberrant cell motility...". However, all through the text, the authors incriminate the GRK2-HDAC6 mediated

tubulin deacetylation as the cause of their observed phenotypes.

The authors should note that the relationship between MT acetylation and stability, presented as a fact all through the manuscript, is a controverted one.
After the publication of the tubulin-deacetylase activity of HDAC6 by Hubbert et al. in Nature (PMID: 12024216) and the claim that cell motility modulated by HDAC6 is due to the control of the acetylation-dependent MT stability, specialists in the field published a brief communication to point out that acetylation does not modulate MT stability (Palazzo et al., Nature, PMID: 12529632). This idea has been since challenged by different groups who showed either an association or no association between MT acetylation and MT stability (see for instance Matsuyama et al., EMBO J, PMID: 12486003 and Zhang et al., MCB, PMID: 18180281).
Facing this situation, the authors cannot build their molecular models on the deacetylation-dependent modulation of MT stability as they do.
It appears to this referee that the modulation of MT acetylation could be used as a readout for the GRK2-mediated regulation of HDAC6 activity, but not as a cause of the observed phenomenon, at least as far as cell motility is concerned.
In contrast, HDAC6-mediated cortactin acetylation has been clearly involved in the control of cell motility (Zhang et al., 2007).

Taking into account the main massage of the manuscript (GRK2/HDAC6 mediated cell spreading and motility) the authors cannot escape from involving cortactin, and therefore should test the modulation of cortactin acetylation by GRK2.

Additionally, given the crucial role of HDAC6 phosphorylation in the GRK2-dependent modulation of HDAC6 activity, it is critical to map this (these) site(s) and to functionally compare it with other published HDAC6 phosphorylation sites. Indeed, recently it has been shown for instance that CK2 also phosphorylates HDAC6 and increases its activity (Watabe & Nakaki, PMID: 21486957), while another work shows that EGFR-mediated phosphorylation of HDAC6 results in reduced deacetylase activity and increased MT acetylation (Deribe et al. PMID: 20029029).
In the studies mentioned above the HDAC6 phosphorylation site has been identified. It becomes therefore here very important to know if the GRK2 site is the same as or different from the CK2 site which is an activator. Additionally, this information would allow, by generating the appropriate mutants, to deepen the functional analyses.

Specific points
1 - All through the manuscript tubulin acetylation is presented as a major determinant in various cellular functions. For the reason presented above, these statements should be largely attenuated. One example is the last paragraph of the introduction: "MT acetylation ...has a prominent role in cell migration... by affecting MT stability and dynamics".

2 - Figure 2C. As far as this referee could understand, the HDAC6 constructs used are from mouse, which does not contain the SE14 domain. This domain should therefore be deleted from the schemes in the figure.

3 - At the bottom of page 6 it is stated that "the second catalytic domain of HDAC6 is the only one that possesses the tubulin-deacetylase activity". The exact nature of the contribution of each of the two catalytic domains of HDAC6 in its substrate-specific activity is controversial. Here again it is not clear on what basis the authors privilege one side of the published data.

Referee #3:

This paper has potential to add significant novelty to the non-receptor targets for GRK2 and involve cell motility and migration through a GRK2-related mechanism via HDAC6 and acetylation of tubulin. It is an interesting study, however there are some issues that need addressing.

1. In general, very few figures have statistically analysis included on their graph. Some of these graphs show data that looks different by eye, but still have decent sized error bars. Since a handful of graphs do show significance statistically, it is confusing for the reader to know if data that does not have explicit statistical analysis mentioned in the legend are significant. For example, Fig. 1A

has been analyzed and shows significance while Fig. 1B either has not been analyzed or does not show significance. Regardless of analysis, the authors present each piece of data as if it is significant. It would be better if the data throughout the papers were consistent. Please comment on the statistical significance of all data.

2. Some western blot images do not match the description of the same figures within the results section. For example, figure 5C shows an increase in HDAC6 expression in the unstimulated cells, which is not addressed in the text. Also, most of the experiments are done in HeLa cells, but the abstract misleads one into thinking that these experiments are done in the more relevant cell lines of fibroblasts and epithelial cells. It would be nice if the experiments in Fig. 6 (cell spreading and cell morphology) could be repeated in more relevant cell lines.

3. It would be helpful and meaningful mechanistically to see data dealing with the significance of the phosphorylation of GRK2 at S670. This is a known ERK site and thus, does ERK activation or ERK inhibition affect the motility processes mediated by GRK2. For example, it would be worthwhile to utilize an activator upstream of ERK and then examine readouts of HDAC6 phosphorylation or immunoprecipitation or tubulin acetylation.

4. It would also be important for this paper to include experiments that examine the phosphorylation state of tubulin by GRK2. Beta-tubulin has been identified as a substrate for GRK2 in a fashion that is also regulated by GPCR activity. The authors should examine changes in tubulin phosphorylation and migration as related to GRK2. Without addressing the interaction between GRK2 and tubulin, it is difficult to determine the full causality of the newly addressed GRK2, HDAC, tubulin pathway.

1st Revision - authors' response 19 October 2011

REVIEWER 1

Answers to major issues

.... Although very interesting, the paper requires attention to three major issues prior to publication:

1.-First, the spreading experiments should be repeated with GRK2-depleted cells.

Following the indication of the reviewer, we have performed additional experiments using GRK2-depleted cells to further substantiate the role of this kinase in cellular spreading.
a) We have monitored by confocal microscopy the spreading of Hela-shGRK2 cells and characterized its morphological features (new Fig S4A). In contrast to the sigmoid increase of cellular spread area with time observed in parental Hela cells, upon GRK2 downregulation cell area increases displaying a pattern similar to that found in Hela-A1 or –K1 cells (that express GRK2 mutants with impaired ability to stimulate HDAC6 deacetylase activity towards tubulin, data shown in Fig. 6). Notably, upon GRK2 depletion the final area of spread cells is smaller than that of control cells (349±18 μm2 in Hela-shGRK2 cells versus 531±36 μm2 in control Hela cells 2h after plating), similar to the results obtained in Hela-A1 and -K1 cells (337±75 and 380±31 μm2, respectively). Furthermore, down-regulation of GRK2 recapitulates the same changes in MT and actin organization (see new Fig. S4B) promoted upon expression of GRK2-K220R or GRK2-S670A mutants (Fig. 6B).
b) We have also used primary cultures of fibroblasts with conditional depletion of GRK2 expression. The spreading of MEFs derived from homozygous GRK2-floxed mice infected with adenovirus expressing Cre-recombinase or a control vector was recorded with the XCELLigence system (new Fig. S7C). Plotted cell index data show consistent changes in the spreading kinetics and progression that are very similar to those obtained in Hela cells upon silencing of GRK2 protein by means of RNA interference. Thus, the time to total spreading of both GRK2-null MEFs and Hela-shGRK2 cells was reduced circa 30% compared to control MEFs and Hela parental cells, respectively, thereby confirming that in different cellular contexts GRK2 downregulation results in an accelerated spreading. Moreover, the extent of tubulin acetylation was similarly increased in both MEFs and Hela cells upon lowering GRK2 protein levels (compare new Fig. 1B and Fig. 1C).

Overall, these results point out that both depletion and functional silencing of GRK2 protein impact on cell spreading and tubulin acetylation in a similar mechanistic manner by means of down-modulation of HDAC6 activity, and also confirm that the GRK2-K220R and –S670A mutants would exert a dominant negative effect in these processes.

2.-Second, the authors must demonstrate how the catalytic activity of GRK2 promotes HDAC6 activity, cell migration and cell spreading. GRK2 may function either by directly binding and phosphorylating (?) HDAC6 or indirectly through an adaptor that in turn is inducing the activity of HDAC6. In this respect it is interesting that the authors found that the GRK2-S670A mutant fails to induce HDAC6-mediated de-acetylation of tubulin. A paper by Pitcher et al. (JBC, 1998) provided evidence that GRK2 associates with and phosphorylates tubulin. It could therefore be that GRK2 forms a ternary complex consisting of tubulin/MTs and HDAC6, phosphorylates tubulin/MTs, which then leads to HDAC6 binding to MTs followed by their de-acetylation? It is not clear which protein is phosphorylated by GRK2! Clearly, this aspect of the paper is not well developed.

Regarding the mechanisms underlying HDAC6 regulation by GRK2, we have performed additional experiments that strongly support that direct binding of GRK2 to and phosphorylation of HDAC6 would promote *per se* the stimulation of HDAC6 activity leading to modulation of cell migration and spreading.

a) The reviewer raises the possibility that the effects of GRK2 on HDAC6 could involve additional adaptors and/or the formation of a ternary complex consisting of GRK2/ tubulin/ and HDAC6. This is ruled out on the basis of several evidences. First, we now show that GRK2 and HDAC6 can directly interact without involvement of any intermediate factor as demonstrated by GST-pull down assays using purified recombinant proteins (new Fig. 2C). In addition, we also find that tubulin and GRK2 compete with each other for binding to HDAC6 in pull down assays (new. Fig. 3A), suggesting that a ternary complex of GRK2/HDAC6/tubulin would not be feasible, thus weakening the possibility that tubulin bound to the kinase might display improved interaction with HDAC6 or become a better substrate for this enzyme. This is consistent with our previous data showing that the kinase-deficient GRK2-K220R mutant (that would preserve scaffolding functions) did not stimulate the ability of GST-HDAC6 to deacetylate brain tubulin *in vitro* (Fig. 3B). An alternative scenario for an indirect effect of GRK2 in which formation of a ternary complex could be dispensable, is that tubulin phosphorylation by GRK2 would enhance its deacetylation. However, we find a similar increase in tubulin acetylation (Fig. 1E) in cells that over-express a GRK2 mutant (GRK2-S670A) that can phosphorylate tubulin but not HDAC6 (Fig. 3D) or express a GRK2 mutant (GRK2-K220R) unable to phosphorylate both proteins. Overall, these data point out GRK2-triggered phosphorylation of HDAC6 and not of tubulin is the relevant event for the effect of this kinase on tubulin deacetylase activity of HDAC6.

b) Further supporting that direct phosphorylation of HDAC6 by GRK2 underlies the observed effects, we already showed that purified GST-HDAC6 is phosphorylated in vitro by recombinant GRK2, and that such phosphorylated HDAC6 displayed higher deacetylase activity towards tubulin (Fig. 2E and 2G of revised version). In addition, we have now identified by site-specific mutagenesis serines 1060, 1062 and 1069 as important phospho-acceptor sites for GRK2 (new Fig.S8A). Cellular assays using these mutants show that phosphorylation of HDAC6 at these residues is necessary for full tubulin deacetylase activity (new Fig. S8B). Moreover, expression of such phosphorylation-deficient mutants failed to mimic the enhanced cell migration promoted by wild type HDAC6, similar to what is noted with the catalytically inefficient HDAC6-DD (new Fig. 7E), and promoted a cell spreading pattern (new Fig. 7F) similar to that observed in the presence of the GRK2 mutants unable to phosphorylate HDAC6 (K220R, S670A) or upon GRK2 downregulation.

3.- *Third, the role of GRK2-mediated activation of HDAC6 and deacetalytion of tubulin needs to be directly shown in migration/spreading assays. This is particularly important since (a) GRK2 can modulate migration in an HDAC6-independent manner (shown in a previous EMBO J paper by the same authors), and (b) HDAC6 can regulate migration and spreading through additional players such as cortactin (Zhang et al., Mol Cell, 2007). It should therefore be shown whether loss of GRK2 and overexpression of catalytically inactive GRK2 impair de-acetalytion of both, cortactin and tubulin, and to which extent each of them contributes to the cellular defects described in the current paper. Furthermore, to which extent does the role of GRK2 on GIT/focal adhesion impact cell migration.*

We agree with the reviewer in that GRK2 and HDAC6 can modulate different cellular processes in a multifaceted way by engaging in different signaling routes with the involvement of both catalytic and scaffolding activities. Following the suggestion of the reviewer, we have directly addressed whether altered GRK2 levels/functionality may affect the activity of HDAC6 towards cortactin. We find that acetylation of endogenous or over-expressed cortactin is not altered by over-expression of wild-type, mutant GRK2 proteins defective in HDAC6 regulation (GRK2-S670A and GRK2-K220R) or upon GRK2 down-regulation (new Fig. S2A-B). Moreover, GRK2-induced migration is not affected by expression of wild-type or cortactin protein mutants that mimic a permanent deaceylated (cortactin-K9R) or acetylated (cortactin-K9Q) state (new Figs. S2C-D). Consistently, tubacin, a specific inhibitor of HDAC6-triggered tubulin deacetylation, counteracts the effect of GRK2 in migration in the absence of changes in cortactin deacetylation (new Figure 1G). These data suggest that tubulin is the relevant target of HDAC6 underlying GRK2-induced migration

Regarding the impact of other components of the GRK2's interactome on cell migration, we are beginning to unveil how GRK2 modulates chemotaxis by means of the concurrent regulation of different migration-related GIT-1 and HDAC6 signalosomes, which would work at different cellular levels to orchestrate cell polarity and focal adhesion dynamics in response to fibronectin. The additional experiments performed might give some clues to estimate the relative "weight" of GIT and HDAC6 contributions in the pro-migratory role of GRK2. In the presence of endogenous GRK2, the HDAC6 inhibitor tubacin promotes circa 40% reduction in migration (new Fig. 1G). Interestingly, cells with extra levels of GRK2 treated with tubacin still display an enhanced migration (80% increase compared to tubacin-treated parental cells), suggesting that a pro-migratory component of GRK2 exist independently of tubulin acetylation. Therefore it is reasonable to propose that tubulin (and cortactin)-independent components of GRK2 related to migration would rely on the modulation of GIT-1 function (Penela et al, 2008). Such contribution could account for 40-45% of the overall effect of GRK2 in migration (compare data in the presence of tubacin in Hela and wt5 cells in the graph of Fig. 1G). In agreement with this scenario, we found that GRK2-induced migration is inhibited by ~ 40% in the presence of a mutant tubulin protein (tubulin-K40A) that abrogates acetylation-deacetylation cycling (new Fig. 1F), while expression of a GIT1 mutant lacking the SHD domain (aa 258–346), which is required for mediating the stimulatory effects of GRK2 on chemotactic signaling (Penela et al., 2008) also results in a migration decay of ~40-45% (Fig. R1 for consideration of the reviewer). Consistently, migration is reduced more extensively when all migratory components of GRK2 (GIT-1 and HDAC6/tubulin-mediated) are simultaneously downplayed by means of inactivation of GRK2 expression (> 85% protein reduction) (new Fig. S1E). Since the relative contribution of a particular GRK2 interacting partner or substrate will depend on the cell type and the specific stimuli that trigger directed motility (discussed in Penela et al, 2009), it would be of interest to explore these processes in other cellular systems and conditions in the future.

4.- *Finally, it is unclear to me why the authors use heterozygous GRK2-null cells in their studies. Floxed mice are published and it should be straight forward to derive cells from the floxed mice and subsequently transduce Cre to obtain true GRK2-null cells for this important study.*

As already mentioned in point 1 above, we have followed the suggestion of the reviewer and Cre-recombinase -infected cells derived from GRK2-floxed mice have been used to downregulate GRK2 expression and further study the role of this protein in tubulin acetylation, migration and spreading (experiments now displayed in Fig 1B, 7B, Fig. S1D-E and Fig. S7C).

Regarding the use of hemizygous GRK2-knockout MEFs, we think that this cellular model is a very useful one to address the role of GRK2 in motility. On one hand, migratory responses are more reproducible in a cellular setting of stable and specific gene inactivation. Alternative cellular approaches based in RNA interference or conditional gene ablation with Cre-lox technology by transfection or infection might cause off-target effects in some occasions. Moreover, since cells from hemizygous GRK2-knockout mice are adapted to permanent down-regulation of GRK2, their differential responsiveness is probably more related to GRK2 levels *per se* and not to collateral disturbances that can be favoured in a context of acute GRK2 protein decay. On the other hand, information from these cells would be of pathophysiological relevance, since such circa 50% variation in GRK2 protein expression is within the range of changes in GRK2 levels reported in a variety of diseases or in diverse tissues at different development stages (Penela et al. 2010 and references therein).

Specific comments:

Fig. S1A-C: GRK2-depletion of wt MEFs reduces GRK2 levels by about 50% while the functional impairments are similar to the GRK2+/- MEFs with a GRK2 reduction of around 75%. I would have expected more pronounced impairments in siRNA-treated GRK2+/- MEFs. How can this discrepancy be explained? Have the siRNA depletions been rescued with siRNA-resistant GRK2 cDNAs?

Regarding the relationship between GRK2 levels and migration, we have performed additional experiments with GRK2-null and GRK2 +/- cells. Decay of GRK2 protein within a range of 40-60% in both shGRK2-infected wild-type MEFs and GRK2 hemizygous MEFs results in a motility reduction of 25-30 %, while a more drastic decrease in GRK2 protein (>85%) leads to a more pronounced effect (60% inhibition) in Cre-infected GRK2-floxed MEFs (new Fig. S1D). The fact that migration was not so strongly inhibited in GRK2+/- MEFs in which GRK2 was also remarkably downregulated (65-70% reduction) by using a shGRK2-RNA construct might be due to differences in the efficacy and stability of protein down-modulation achieved by RNA-interference vs gene-depletion. The possibility that such defective locomotion might be due to off-target effects of the shGRK2-RNA construct is remote, as a distinct approach for GRK2 depletion also results in defective migration. On the other hand, the possibility that there is a threshold value of GRK2 levels below which no further decrease in cell migration would be noted could be considered.

Fig. 1C: is the NaB effect on wt cells significant?

The subtle effects of NaB (now Fig.1D) are not significant in wild-type cells or in GRK2 +/- MEFs. Statistical analyses are now mentioned in the figure legends throughout the manuscript, remarking significance when appropriate.

Fig. 1D: overexpression of wt GRK2 does not affect tubulin acetylation, while it significantly affects migration in a wound closure assay. The authors argue that localized increases in GRK2 activity may explain these effects. Is it possible that migration represents an activation signal of GRK2 which is missing in resting cells? What could such a signal be?

Our results indicate that localized increases in tubulin deacetylation elicited by an activated pool of GRK2 are involved in the pro-migratory effect of GRK2. We have demonstrated in a previous report that membrane recruitment of GRK2 is critical for this process (Penela et al., 2008). In such specific locations GRK2 not only will participate in the modulation of GIT1 functionality in a scaffolding-dependent manner, but also in the stimulation of tubulin deacetylation through the phosphorylation of HDAC6. In support of this model, we find that total GRK2 is enriched at the leading edge of wounded-motile cells, where HDAC6 is also accumulated (Fig 4 and new Fig. S3A). Moreover, our data indicate that phosphorylation of GRK2 at the S670 regulatory site would act as a key switch that specifically modulates its ability to phosphorylate HDAC6 and thus to affect its activity. Importantly, we now show that GRK2 is prominently phosphorylated at S670 in the leading edge as detected by confocal microscopy (new Fig. S3B) adding to our previous biochemical analysis of the pseudopodia of motile cells by immunoblot (Fig.5C). Consistent with the increased ability of pS670-GRK2 to regulate HDAC6, tubulin is prominently deacetylated in the leading edge and pseudopodia of migrating cells. In agreement with the reviewer's suggestion that migration must provide an activation signal for GRK2 in order to modulate HDAC6 and tubulin deacetylation, we have included new data showing that a potent chemotactic stimuli as EGF triggers a rapid and sustained phosphorylation of endogenous GRK2 at S670, which is paralleled by an active deacetylation of steady-state levels of tubulin. In the presence of such migratory stimuli, the extent of pS670-GRK2 and tubulin deacetylation is clearly enhanced in the presence of extra GRK2 (new Fig. 3E).

Fig. 2A-C: the assays do not rule out the possibility that HDAC6 immunoprecipitates GRK2 through its ability to bind tubulin. It has been shown previously that GRK2 can also bind tubulin. Can GRK2 bind HDAC6 in the absence of tubulin? Is HDAC6 binding and phosphorylation by GRK2 a tubulin-dependent or -independent event?

We agree with the reviewer that co-immunoprecipitation assays (shown in previous Figs. 2A-C, now shifted to Figs. 2A-B, D) do not rule out a role for tubulin in the association of GRK2 with HDAC6. As detailed in our answer to point 2 above, we have analyzed in more detail the interaction of these

proteins by using in vitro binding assays in order to address the specific questions raised by the reviewer. Our data indicate that recombinant GRK2 can bind purified GST-HDAC6 in the absence of tubulin (new Fig. 2C). Moreover, a ternary protein complex composed of tubulin/HDAC6/GRK2 is not feasible because tubulin competes with GRK2 for binding to HDAC6 in pull down assays (new Fig. 3A). In addition, we previously showed that recombinant GRK2 is able to phosphorylate purified HDAC6 in the absence of tubulin (Fig. 2D and 3D). Therefore, we can conclude that HDAC6 binding and phosphorylation by GRK2 can occur independently of tubulin.

Fig.2F: can the phosphor-acceptor site in DD2 be identified? Does DD2 contain a GRK2 phospho-consensus site? Such an identification and subsequent mutation of the acceptor site would tremendously strengthen the impact of the paper and the observation that the formation of the ternary complex consisting of GRK2/tubulin/HDAC6 is kinase dependent.

As detailed in our answer to point 2 b) above, we have now identified by site-specific mutagenesis serines 1060, 1062 and 1069 as important phospho-acceptor sites for GRK2 (new Fig.S8A). Although there is no defined GRK2 phospho-consensus site, structural data of its kinase domain predicts preferential targeting of sites with acidic amino acids N-terminal to the phosphorylated residue and hydrophilic residues at P+1 (Lodowski DT et al., 2003). With this criterion we have engineered a battery of single, double or triple mutations to alanine to target potential serine/threonine residues within the second half of HDAC6. We find that phosphorylation by GRK2 is clearly decreased when residues S1060, S1062 on HDAC6 are simultaneously mutated (HDAC6-S1060/1062A). Moreover, mutation of S1069 on the double mutant HDAC6-S1060/1062A further reduces the phosphorylation (new Fig. S8A). The novel sites phosphorylated by GRK2 on HDAC6 localize in the region between the second catalytic domain (DD2) and the ubiquitin binding domain of HDAC6, suggesting an indirect regulation of catalytic DD2 domain activity through allosteric conformational changes or altered responsiveness to HDAC6 activator/inhibitors. Indeed, modulation of tubulin deacetylase activity has been reported as a result of phosphorylation events both within and out of the DD2 domain (Deribe et al., 2009; Watabe and Nakaki, 2011). Importantly, cellular assays using these mutants show that phosphorylation of HDAC6 at these residues is necessary for full tubulin deacetylase activity (new Fig. S8B). Moreover, expression of such phosphorylation-deficient mutants failed to mimic the enhanced cell migration promoted by wild type HDAC6, similar to what is noted with the catalytically inefficient HDAC6-DD (new Fig. 7E), and promoted a cell spreading pattern (new Fig. 7F) similar to that observed in the presence of the GRK2 mutants unable to phosphorylate HDAC6 (K220R, S670A) or upon GRK2 downregulation.

Fig. 3D: the GRK2-S670A reduces HDAC6 phosphorylation. Does the pospho-mimicking mutation S760D enhance phosphorylation?

In order to answer the question raised by the reviewer, we have performed in vitro kinase assays with purified GST-HDAC6 protein and immunoprecipitated GRK2 mutants that are either defective (GRK2-S670A) or mimic (GRK2-S670D) phosphorylation at S670 (Fig R2 for consideration by the reviewer). Contrary to that observed with GRK2-S670A, the S670D is able to efficiently phosphorylate HDAC6 compared to a control of kinase-inactive GRK2 protein overexpressed and immunoprecipitated at similar levels. As GRK2 exists as a phosphoprotein in cells with a variable but remarkable level of pS670 (Pitcher JA et al., 1999), the wild-type protein was not included in this assay for clarity.

Fig. 4: the images are of poor quality. HDAC6 accumulation at the leading edge is only visible in a single cell of the low magnification image. This is not convincing and has to be improved. In my view HDAC6 levels do not have to be increased at the leading edge. Also an evenly distributed HDAC6 will show an uneven activity if itsactivators/inactivators are unevenly distributed. Furthermore, the scratch should be indicated with a dotted line.

In response to this point, we have modified the confocal images in order to show more clearly the accumulation of HDAC6 at the leading edge of motile cells (new panels of zoomed cells, Fig. 4). Additional cellular fields have been incorporated that not only reflect the presence of GRK2 and HDAC6 at cell directional protrusions devoid of acetylated tubulin (new Fig.S3A), but also co-localization of both HDAC6 and phosphorylated GRK2 at S670 at these regions (new Fig. S3B). We agree with the reviewer that a graded or uneven activity of HDAC6 might be achieved only by an unequal presence of its regulators. In this regard, both the recruitment of GRK2 and its higher

phosphorylation at S670 in the leading front of polarized cells could be sufficient to stimulate HDAC6 activity. But, in addition, we also observed an increased signal for HDAC6 protein in this location. Such preferential accumulation of HDAC6 could contribute not only to up-regulate its deacetylase activity but also favor scaffolding activities of relevance in migration (such as the interaction with EB1). Nevertheless, HDAC6 accumulation at the leading edge has been consistently reported by others groups in a variety of cellular types and under different migratory stimuli. (Li et al., 2011 Protein Cell; Kaluza et al., 2011 EMBO J; Hubbert et al., 2002 Nature).

We would like to thank the reviewer for his/her thoughtful comments and suggestions that have helped to improve the manuscript.

REVIEWER 2

General comments

1.-It seems that the authors have been confused by the widely used HDAC6 qualifier, "tubulin-deacetylase", which somehow shadows the fact that HDAC6 is primarily a protein deacetylase and tubulin is only one of its substrates. This leads the authors to interpret all their findings through the modulation of tubulin acetylation, while some of their observations would better fit with the acetylation of another HDAC6 substrate, cortactinThe authors seem to be aware of this fact since in the discussion section, the possibility of a connection to other HDAC6 substrates is mentioned at least twice.
One example is at the end of the discussion section, where we can read : "...GRK2 by increasing the HDAC activity of HDAC6 towards a-tubulin (AND PERHAPS TOWARD OTHER SUBSTRATES) would favour aberrant cell motility...". However, all through the text, the authors incriminate the GRK2-HDAC6 mediated tubulin deacetylation as the cause of their observed phenotypes.

The authors should note that the relationship between MT acetylation and stability, presented as a fact all through the manuscript, is a controverted one.
After the publication of the tubulin-deacetylase activity of HDAC6 by Hubbert et al. in Nature (PMID: 12024216) and the claim that cell motility modulated by HDAC6 is due to the control of the acetylation-dependent MT stability, specialists in the field published a brief communication to point out that acetylation does not modulate MT stability (Palazzo et al., Nature, PMID: 12529632). This idea has been since challenged by different groups who showed either an association or no association between MT acetylation and MT stability (see for instance Matsuyama et al., EMBO J, PMID: 12486003 and Zhang et al., MCB, PMID: 18180281).
Facing this situation, the authors cannot build their molecular models on the deacetylation-dependent modulation of MT stability as they do.
It appears to this referee that the modulation of MT acetylation could be used as a readout for the GRK2-mediated regulation of HDAC6 activity, but not as a cause of the observed phenomenon, at least as far as cell motility is concerned. In contrast, HDAC6-mediated cortactin acetylation has been clearly involved in the control of cell motility (Zhang et al., 2007).

Taking into account the main massage of the manuscript (GRK2/HDAC6 mediated cell spreading and motility) the authors cannot escape from involving cortactin, and therefore should test the modulation of cortactin acetylation by GRK2.

We agree with the reviewer in that the deacetylase activity of HDAC6 affects a growing repertoire of substrates, with tubulin and cortactin being the most extensively characterized. Although both tubulin and cortactin have been implicated in the migration process, their contribution to the effect of HDAC6 on migration is still an open question. On the basis of the evidences compiled until now in favour or not of a role of tubulin (Wang et al. 2010, Ann Biomed Eng; Wu et al. 2010, J. Cell. Biol.; Creppe et al., 2009, Cell; Wang et al., 2010, Invest Ophthalmol Vis Sci; to name but a few) or cortactin (Kaluza et al., 2011, EMBO J; Rey et al., 2011, Eur J Cell Biol; Tsunoda et al., 2011, J Invest Dermatol; Zhang et al., 2007 , Mol Cell, to name but a few) in the HDAC6-dependent migration of different cellular types, we believe it is not straightforward to assert which substrate (tubulin or cortactin) is more relevant for the pro-migratory function of HDAC6. On top of that, it has been reported that HDAC6-induced migration does not require deacetylase activity in some cellular types (Cabrero et al., 2006 Mol Biol Cell). Therefore, it is likely that the mechanisms

underlying the effect of HDAC6 on migration will depend on the cell type and the specific signaling routes activated by chemotactic stimuli. In such diverse scenarios, it is feasible that a different set of HDAC6 modulators could be activated, promoting distinct functional consequences for different HDAC6's substrates.
In this context, following the suggestion of the reviewer, we have directly addressed whether altered GRK2 levels/functionality may affect the activity of HDAC6 towards cortactin. As detailed below, our additional experiments indicate that GRK2 stimulates selectively the deacetylase activity of HDAC6 towards tubulin (not cortactin) in a kinase-dependent manner, based on the differential effects of specific tubulin-deacetylase vs general deacetylase inhibitors of HDAC6, the evaluation of the impact of GRK2 mutants with defective HDAC6 regulation on cortactin deaceylation and the sensitivity of GRK2-induced migration to cortactin deacetylation.
We find that tubacin (a specific inhibitor of HDAC6-dependent deacetylation of tubulin that not modifies the extent of cortactin acetylation) counteracts the effect of GRK2 in migration in the absence of changes in cortactin deacetylation (new Figure 1G). Further stressing this point, acetylation of endogenous or over-expressed cortactin is not altered by over-expression of wild-type, mutant GRK2 proteins defective in HDAC6 regulation (GRK2-S670A and GRK2-K220R) or upon GRK2 down-regulation (new Fig. S2A-B). Interestingly, expression of extra cortactin-wt or cortactin-K9R (which mimics the deacetylation state) stimulated migration of Hela cells, whereas they failed to increase further the higher motility of Hela-wt5 cells (new Fig.S2C-D). Moreover, migration of Hela but not of Hela-wt5 cells was inhibited in the presence of cortactin-K9Q (which mimics acetylation) (new Fig. S2D), demonstrating that GRK2 regulates migration independently of the deacetylation status of cortactin. Overall, these data suggest that tubulin and not cortactin is the relevant target of HDAC6 underlying GRK2-induced migration.

The reviewer also poses the intricate issue of whether tubulin acetylation modifies MT stability or such modification serves unrelated functions and merely accumulates in long-lived MTs. We agree that this issue is controversial and that there are not enough supporting evidences to claim that deacetylation of tubulin is required directly or indirectly for the maintenance of dynamic MTs, despite those pioneer MTs at the lamellipodium of motile cells (which instability and adjustable growth is instrumental for motility) are fully deacetylated. Therefore, we have included a sentence in the Introduction section clearly remarking this controversy (final paragraph in the first page of the Introduction).
It is interesting to note, however, that a recent report has indicated that acetylation of a-tubulin is related to the posttranscriptional fine-tuning of a-tubulin levels and to the dynamics of polymerization and depolymerization (Solinger et al., 2011 PLoS Genetics). In this context, the incidence of shortening and shrinkages events would be higher in deacetylated MTs as a result of increased a-tubulin protein turnover in agreement with previous reports (Tran et al., 2007 J Cell Sci.; Matsuyama et al., 2002 EMBOJ). On the other hand, acetylation/deacetylation of tubulin is well demonstrated to affect loading and transport of cargo vesicles along microtubules. An asymmetric distribution of acetylated and stabilized MTs is key to the deliverance of components to the leading edge (lamella MTs are hiper-acetylayed), thereby guaranteeing stable cell polarity in motile cells.
Therefore, acetylation/deacetylation cycling of tubulin (and underpinning regulatory processes) is concerned with motility in several ways. In this regard, we have performed additional experiments that stress the relevance of dynamic tubulin deacetylation in migration. Abrogation of acetylation-deacetylation cycling by means of expression of a tubulin mutant defective in acetylation (tubulin-K40A) results in circa 35-40% inhibition of GRK2-induced migration (new Fig. 1F). A forced reduction in tubulin acetylation impinges cellular morphology in neurons, with loss of polarity (rounded cells) and motility (Creppe et al., 2009), whereas artificial hyper-acetylation results in similar phenotypic alterations in neurons (Tapia et al., 2010, PLoS One). These findings suggest that a fine-tuning dynamic control and spatial asymmetry of acetylation/deacetylation is required for effective protrusive activity during locomotion.
Our data are consistent with the concept that GRK2 regulation of HDAC6 deacetylase activity towards tubulin (and not cortactin) could favour the presence of MTs prone to instability and remodelling at specific cellular regions that integrate chemotactic information, wherein they could impact polarity signaling pathways and/or adhesion.

2.-Additionally, given the crucial role of HDAC6 phosphorylation in the GRK2-dependent modulation of HDAC6 activity, it is critical to map this (these) site(s) and to functionally compare it with other published HDAC6 phosphorylation sites. Indeed, recently it has been shown for instance that CK2 also phosphorylates HDAC6 and increases its activity (Watabe & Nakaki, PMID:

21486957), while another work shows that EGFR-mediated phosphorylation of HDAC6 results in reduced deacetylase activity and increased MT acetylation (Deribe et al. PMID: 20029029).
In the studies mentioned above the HDAC6 phosphorylation site has been identified. It becomes therefore here very important to know if the GRK2 site is the same as or different from the CK2 site which is an activator. Additionally, this information would allow, by generating the appropriate mutants, to deepen the functional analyses.

As suggested by the reviewer, we have addressed the identification of GRK2 phosphorylation sites on HDAC6. We have identified by site-specific mutagenesis serines 1060, 1062 and 1069 as important phospho-acceptor sites for GRK2 (new Fig.S8A). Although there is no defined GRK2 phospho-consensus site, structural data of its kinase domain predicts preferential targeting of sites with acidic amino acids N-terminal to the phosphorylated residue and hydrophilic residues at P+1 (Lodowski DT et al., 2003). With this criterion we have engineered a battery of single, double or triple mutations to alanine to target potential serine/threonine residues within the second half of HDAC6. We find that phosphorylation by GRK2 is clearly decreased when residues S1060, S1062 on HDAC6 are simultaneously mutated (HDAC6-S1060/1062A). Moreover, mutation of S1069 on the double mutant HDAC6-S1060/1062A further reduces the phosphorylation (new Fig. S8A).
Sequences phosphorylated by GRK2 can share some structural determinants with the preferred sites of other kinases such as CKII, and it is not unlikely that these kinases may phosphorylate the same residues in a certain substrate. However, mutation of the site targeted by CKII on HDAC6 (HDAC6-S458A, Watabe & Nakaki, 2011) does not affect the ability of GRK2 to fully phosphorylate HDAC6 (Fig. S8A).
The novel GRK2-phosphorylated sites on HDAC6 localize in the region between the second catalytic domain (DD2) and the ubiquitin binding domain of HDAC6, suggesting an indirect regulation of catalytic DD2 domain activity through allosteric conformational changes or altered responsiveness to HDAC6 activator/inhibitors. Indeed, modulation of tubulin deacetylase activity has been reported as a result of phosphorylations events both within and out of the DD2 domain (Deribe et al., 2009; Watabe and Nakaki, 2011). Importantly, cellular assays using our novel mutants show that phosphorylation of HDAC6 at these residues is necessary for full tubulin deacetylase activity (new Fig. S8B). Moreover, expression of such phosphorylation-deficient mutants failed to mimic the enhanced cell migration promoted by wild type HDAC6, similar to what is noted with the catalytically inefficient HDAC6-DD (new Fig. 7E), and promoted a cell spreading pattern (new Fig. 7F) similar to that observed in the presence of the GRK2 mutants unable to phosphorylate HDAC6 (K220R, S670A) or upon GRK2 downregulation. Overall, our data support the notion that GRK2-mediated phosphorylation of HDAC6 enhanced its deacetylase activity towards tubulin and lead to changes in cell migration and spreading patterns.

Specific points
1 - All through the manuscript tubulin acetylation is presented as a major determinant in various cellular functions. For the reason presented above, these statements should be largely attenuated. One example is the last paragraph of the introduction: "MT acetylation ...has a prominent role in cell migration... by affecting MT stability and dynamics".

As discussed in detail above under point 1, although our data are consistent with a major involvement of tubulin deacetylation in the effects of GRK2 in cell migration and spreading, we have modified the sentence in the Introduction as indicated by the reviewer, and some references to other potential deacetylase-dependent and independent functions of GRK2-activated HDAC6 have been included in the Discussion section.

2 - Figure 2C. As far as this referee could understand, the HDAC6 constructs used are from mouse, which does not contain the SE14 domain. This domain should therefore be deleted from the schemes in the figure.

The reviewer is right and the schemes in the figure 2D have been amended

3 - At the bottom of page 6 it is stated that "the second catalytic domain of HDAC6 is the only one that possesses the tubulin-deacetylase activity". The exact nature of the contribution of each of the two catalytic domains of HDAC6 in its substrate-specific activity is controversial. Here again it is not clear on what basis the authors privilege one side of the published data.

We agree in that the exact nature of the contribution of each of the two catalytic domains of HDAC6 (hdac1 and hdac2) in its substrate-specific activity is controversial in the literature. However, a larger number of reports support that the second deacetylation domain is more critically involved in tubulin deacetylation , on the basis of the effect of single mutations in either of the hdac domains versus double mutations on the deacetylation of different substrates (reviewed in Kaluza et al., 2011, EMBO J), and the compared inhibitory actions of the general , non-selective deacetylase inhibitor TSA with tubacin, a compound that specifically interacts with the second hdac domain and strongly prevents tubulin deacetylation, whereas displaying a mild effect on the global deacetylation activity of HDCA6 (Haggarty et al., 2003 PNAS). Such arguments are now briefly mentioned to modify and make more clear the sentence noted by the reviewer. On top of that, our data showing that GRK2 phosphorylation of HDAC6 takes place in the second half of the protein is consistent with the idea that this modification would preferentially modulate the activity of the second catalytic domain and specifically enhance tubulin-deacetylase activity. The fact that cortactin-deacetylase activity is not affected by GRK2 phosphorylation of HDCA6 further support this interpretation.

We would like to thank the reviewer for his/her suggestions that have helped to improve the manuscript.

REVIEWER 3

This paper has potential to add significant novelty to the non-receptor targets for GRK2 and involve cell motility and migration through a GRK2-related mechanism via HDAC6 and acetylation of tubulin. It is an interesting study, however there are some issues that need addressing.

1. In general, very few figures have statistically analysis included on their graph. Some of these graphs show data that looks different by eye, but still have decent sized error bars. Since a handful of graphs do show significance statistically, it is confusing for the reader to know if data that does not have explicit statistical analysis mentioned in the legend are significant. For example, Fig. 1A has been analyzed and shows significance while Fig. 1B either has not been analyzed or does not show significance. Regardless of analysis, the authors present each piece of data as if it is significant. It would be better if the data throughout the papers were consistent. Please comment on the statistical significance of all data.

Following the suggestion of the referee, statistical analyses are now mentioned in the figure legends throughout the manuscript, remarking significance when appropriated. In some instances (Figs.1G, 7C) individual data of a representative experiment are plotted, following the advice of the Editor.

2. Some western blot images do not match the description of the same figures within the results section. For example, figure 5C shows an increase in HDAC6 expression in the unstimulated cells, which is not addressed in the text. Also, most of the experiments are done in HeLa cells, but the abstract misleads one into thinking that these experiments are done in the more relevant cell lines of fibroblasts and epithelial cells. It would be nice if the experiments in Fig. 6 (cell spreading and cell morphology) could be repeated in more relevant cell lines.

Following the suggestion of the reviewer, we have performed some additional experiments using primary fibroblasts cultures with conditional depletion of GRK2 expression. MEFs derived from homozygous GRK2-floxed mice infected with adenovirus expressing Cre-recombinase or a control vector to obtain a significant downregulation (circa 85%) of GRK2 protein levels (new Fig. S1E). The extent of tubulin acetylation was similarly increased in both MEFs and Hela cells upon lowering GRK2 protein levels (compare new Fig. 1B and Fig. 1C), in parallel with a reduced motility caused by such decrease in GRK2 levels (see new Fig. S1D). In order to complement data in Fig. 6 as pointed by the reviewer, the spreading of MEFs derived from homozygous GRK2-floxed mice infected with adenovirus expressing Cre-recombinase or a control vector was recorded with the XCELLigence system (new Fig. S7C). Plotted cell index data show consistent changes in the spreading kinetics and progression that are very similar to those obtained in Hela cells upon silencing of GRK2 protein by means of RNA interference. Thus, the time to total spreading of both GRK2-null MEFs (new Fig. 7B) and Hela-shGRK2 cells was reduced circa 30% compared to control MEFs and Hela parental cells, respectively, thereby confirming that in different cellular

contexts GRK2 downregulation results in an accelerated spreading. We have also checked the manuscript for adequate description of the figures.

3. It would be helpful and meaningful mechanistically to see data dealing with the significance of the phosphorylation of GRK2 at S670. This is a known ERK site and thus, does ERK activation or ERK inhibition affect the motility processes mediated by GRK2. For example, it would be worthwhile to utilize an activator upstream of ERK and then examine readouts of HDAC6 phosphorylation or immunoprecipitation or tubulin acetylation.

Our data indicate that phosphorylation of GRK2 at the S670 regulatory site would act as a key switch that specifically modulates its ability to phosphorylate HDAC6 and thus to affect its activity. Importantly, we now show that GRK2 is prominently phosphorylated at S670 in the leading edge as detected by confocal microscopy (new Fig. S3B) adding to our previous biochemical analysis of the pseudopodia of motile cells by immunoblot (Fig.5C). Consistent with the increased ability of pS670-GRK2 to regulate HDAC6, tubulin is prominently deacetylated in the leading edge and pseudopodia of migrating cells.
On the other hand, in agreement with the reviewer's suggestion, we have included new data showing that a potent chemotactic stimuli and ERK activator as EGF triggers a rapid and sustained phosphorylation of endogenous GRK2 at S670, which is paralleled by an active deacetylation of steady-state levels of tubulin. In the presence of such migratory stimuli, the extent of pS670-GRK2 and tubulin deacetylation is clearly enhanced in the presence of extra GRK2 (new Fig. 3E).
4. It would also be important for this paper to include experiments that examine the phosphorylation state of tubulin by GRK2. Beta-tubulin has been identified as a substrate for GRK2 in a fashion that is also regulated by GPCR activity. The
authors should examine changes in tubulin phosphorylation and migration as related to GRK2. Without addressing the interaction between GRK2 and tubulin, it is difficult to determine the full causality of the newly addressed GRK2, HDAC, tubulin pathway.

To address the point raised by the reviewer regarding a potential role for GRK2 interaction/phosphorylation with tubulin in the observed effects, we have performed additional experiments that strongly support that direct binding of GRK2 to and phosphorylation of HDAC6 would promote *per se* the stimulation of HDAC6 activity leading to modulation of cell migration and spreading.
One possibility was that the effects of GRK2 on HDAC6 could involve the formation of a ternary complex consisting of GRK2/ tubulin/ and HDAC6. This is ruled out on the basis of several evidences. First, we now show that GRK2 and HDAC6 can directly interact without involvement of any intermediate factor as demonstrated by GST-pull down assays using purified recombinant proteins (new Fig. 2C). In addition, we also find that tubulin and GRK2 compete with each other for binding to HDAC6 in pull down assays (new. Fig. 3A), suggesting that a ternary complex of GRK2/HDAC6/tubulin would not be feasible, thus weakening the possibility that tubulin bound to the kinase might display improved interaction with HDAC6 or become a better substrate for this enzyme. This is consistent with our previous data showing that the kinase-deficient GRK2-K220R mutant (that would preserve scaffolding functions) did not stimulate the ability of GST-HDAC6 to deacetylate brain tubulin *in vitro* (Fig. 3B).
An alternative scenario for an indirect effect of GRK2 in which formation of a ternary complex could be dispensable, is that tubulin phosphorylation by GRK2 would enhance its deacetylation. However, we find a similar increase in tubulin acetylation (Fig. 1E) in cells that over-express a GRK2 mutant (GRK2-S670A) that can phosphorylate tubulin but not HDAC6 (Fig. 3D), or express a GRK2 mutant (GRK2-K220R) unable to phosphorylate both proteins.
Further supporting that direct phosphorylation of HDAC6 by GRK2 underlies the observed effects of the kinase in cell migration and spreading, we have now identified by site-specific mutagenesis serines 1060, 1062 and 1069 as important phospho-acceptor sites for GRK2 (new Fig.S8A). Cellular assays using these mutants show that phosphorylation of HDAC6 at these residues is necessary for full tubulin deacetylase activity (new Fig. S8B). Moreover, expression of such phosphorylation-deficient mutants failed to mimic the enhanced cell migration promoted by wild type HDAC6, similar to what is noted with the catalytically inefficient HDAC6-DD (new Fig. 7E), and promoted a cell spreading pattern (new Fig. 7F) similar to that observed in the presence of the GRK2 mutants unable to phosphorylate HDAC6 (K220R, S670A) or upon GRK2 downregulation. Overall, these data point out GRK2-triggered phosphorylation of HDAC6 and not of tubulin is the relevant event for the observed effects of this kinase.

We would like to thank the reviewer for his/her positive comments and suggestions that have helped to improve the manuscript.

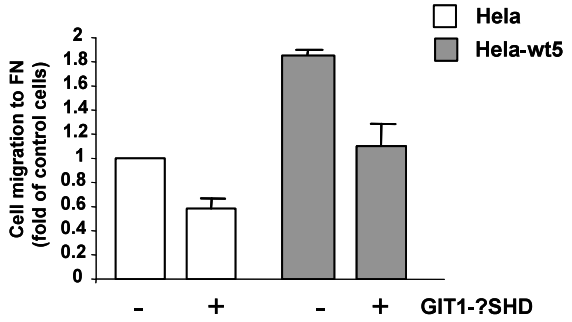

Figure R1. Effect of GRK2 on fibronectin-induced cell motility is partially mediated by GIT-1. Control Hela cells or cells stably overexpressing GRK2wt were transiently co-transfected with CD8 antigen and GIT1-ΔSHD or empty vector. Cells positive for CD8 expression were sorted for migration assays by using microbeads precoated with anti-CD8 antibody. Chemotactic motility to FN was assessed as detailed in Material and methods. Data are mean±SEM of 2 independent experiments performed in duplicate.

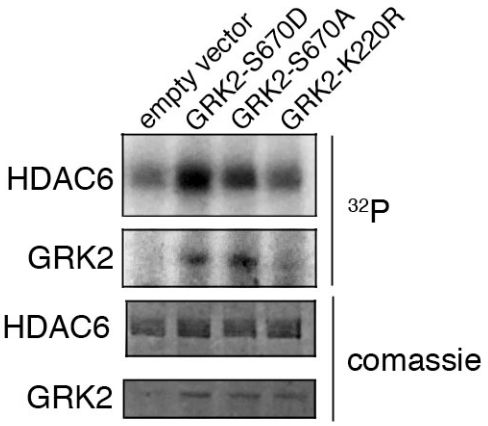

Figure R2. Phosphorylation of GRK2 on S670 modulates its kinase activity towards HDAC6. Different constructs of GRK2 (the catalytic-dead GRK-K220R mutant or mutants that are either defective (GRK2-S670A) or mimic (GRK2-S670D) phosphorylation at S670) were transiently over-expressed in HEK-293 cells and immunoprecipitated with a specific anti-GRK2 antibody. Phosphorylation of GST-HDAC6 (100 nM) was performed in the presence of [γ-^{32}P] -ATP using either immunoprecipitated GRK2-S670A, GRK2-S670D or GRK2 K220R proteins, followed by SDS-PAGE and autoradiography. Protein levels of HDAC6 and GRK2 were monitored by Coomassie staining. Data representative from 2-3 independent experiments are shown

2nd Editorial Decision 11 November 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2011-78032R. It has now been seen by all three referees, whose comments are enclosed below. As you will see, the referees are happy with the revision, and now fully support publication in EMBOJ. I am therefore pleased to tell you that we will be able to accept your manuscript for publication here. However, I do just have a few questions about the figures that I need you to deal with first.

- Several of the Western blots seem very highly contrasted (notably in figures 1B, C, 5 B, C and

7C). In general, we ask that only moderate contrast adjustments are applied (so that background is still visible), and I would ask that you replace these panels with less contrasted versions.

- In figure 3C, there are some aberrations - lines across the gels above and/or below the bands (see attached figure): these may well be the result of cropping, but I'm sure you agree that it looks a little odd, and I would ask you to take a look at these and replace the panels.

- In both these cases, I need to ask you for the original, unprocessed blots which we require for our records. Moreover, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide the original, uncropped and unprocessed scans of all gels used in the figures (or at least of key data panels)? These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential.Ideally, we would ask for separate files for each figure panel, which you can upload as a single zip file as "source data" - via EJP. These will then appear as supplementary files and be directly linked to each relevant main figure.

Please let me know if you have any questions about this; otherwise I look forward to receiving the final version of your manuscript soon.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

I am fully satisfied with the rebuttal. Congratulations to this nice paper!

Referee #2

Data are now mostly clear-cut and convincing and most of the questions and concerns raised previously have been taken into account. I can therefore recommend this manuscript for publication.

Referee #3

The authors have addressed previous concerns and i think this is an exciting and important study.

2nd Revision - authors' response 17 November 2011

Enclosed please find the final version of our manuscript entitled “A novel GRK2/HDAC6 interaction modulates cell spreading and motility” (EMBOJ-2011-78032R1) by Vanesa Lafarga, Ivette Aymerich, Olga Tapia, Federico Mayor, jr. and Petronila Penela.

According to the points raised in your decision letter of November 11, several blots have been replaced as indicated and corresponding original, unprocessed chemiluminescent-developed films and infrared Odissey imaging files are provided.

1) Contrast and brightness have been adjusted in the blots of Figures 1B, 1C, 5B, 5C and 7C in order to make visible some background. It must be noted that infrared detection due to its high sensitivity gives significantly less background signal than chemiluminescence in Figures1B-C, 5B-C, 3E (Ac-Tubulin and pERK blots) or 7C, what may result in images with a “contrasted” appearance. Original blots of these panels have been scanned and provided.

2) Flawed panels in figure 3E have been replaced.

Following the indication of the editor, we have also provided the source data of the most relevant blots of selected panels in Figure 1E, 1G, 2C, 2G, 3G and 3D.

We hope that with these modifications our manuscript will be acceptable now for publication in the EMBO Journal.

