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Group I and II mammalian PAKs have different modes of activation by Cdc42

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 14 November 2011

Thank you for your patience while your study has been under peer-review at EMBO reports. We have now received the enclosed reports from the three referees that were asked to assess it. As you will see, none of the referees are very positive about the manuscript. Although they appreciate the interest of the topic, they also raise numerous concerns regarding the conclusiveness and quality of the data presented. In addition, referees 1 and 3 criticize the overall presentation and organization of the study, and referee 3 suggests it would be better suited to publication as a full-length article elsewhere.

Given these opinions and the fact that, due to pressure for space, EMBO reports can only invite revision of papers that receive enthusiastic support from a majority of referees, I am afraid that we cannot offer to publish your manuscript.

I am sorry to disappoint you on this occasion, and hope that the referee comments are helpful in your continued work in this area.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In this paper the authors present interesting new findings about the protein kinase PAK4. Specifically they identify new splice variants of PAK4, which have internal deletions, they find that PAK4 contains an auto inhibitory domain, and they find that the activation loop Ser474 is constitutively phosphorylated and not regulated by external factors, and finally they find that binding of Cd42 activates PAK4 but that the isolated PAK4 has no memory of this state. These are all interesting and novel findings, but do not always seem to fit together into one coherent story. The paper could be better organized, and there is some data does not seem to add much to the main theme of the paper. Furthermore, the paper should be edited for writing and grammar, so that it reads more clearly, and this could alleviate some of the problems described above. Some specific comments are below.

1. In the Introduction section the authors discuss the role for PAK4 in cancer. PAK4 also has a number of other important functions, however, including roles in development. These are not addressed.

2. In the first paragraph of the results section the sentence "Human PAK4 (591 residues) is a ubiquitous kinases; it transpires a mRNA encoding a smaller PAK4 is relatively abundant" does not make sense. This sentence should be clarified and the entire manuscript should be carefully edited.

3. Figure 1B. The finding of a shorter PAK4a transcript is interesting and supported by genome sequencing. However, the data in the western is not entirely clear. Flag tagged full length PAK4 (PAK4a) is bigger than endogenous PAK4 as is expected, but flag tagged PAK4b seems to be smaller than the indicated endogenous protein. Why is this? And why is the band in the B16 lane smaller than the one in the N1E lane. Is this PAK4c? Also, the flag tagged and endogenous proteins should be run on the same gel here, for size comparisons. The finding of the internal deletion mutant is interesting, but does not seem to lend strong support for the presence of an autoinhibitory domain, and does not really seem to add much to the paper.

4. Figure 1C. Why does the catalytically active PAK4 have strong activity even though it has the later described auto inhibitory domain? It would be interesting if the authors also looked at the PAK4(S474E) mutant here. The results could strengthen their argument. Also in the figure, the addition of Cdc42 seems to lead to a slight increase in Raf phosphorylation in response to expression of the full length protein. Why is this? This is not addressed.

5. In Figure 2B as well as 1C, the authors look at p32 labeling as an indication of autophosphorylation. However, it is not clear which sites are being phosphorylated. More discussion or speculation about this would be helpful. Also in 2C, the delta50PAK4b seems to have a low autophosphorylation level but a high level of Raf phosphorylation. How can this be explained? This should be addressed.

6. Figure 4B. This figure was the most troubling. The authors show that S474 phosphorylation does not correlate with treatment with a number of stimuli including serum and sorbitol. However, how do we know that those are even activators of PAK4? The positive control is p38, but that does not mean that PAK4 is activated by these stimuli. Without another way to look at PAK4 activation, these experiments area not that useful. What about looking at PAK4 autophosphorylation here? Furthermore, 90 minute serum starvation is probably not sufficient for examining serum stimulation, especially in a tumor cell. Why was only once cell type chosen for these experiments? Regarding the timing of serum stimulation, some pathways may be activated quite transiently, within 1 - 2 minutes, so that a more detailed time course could also more useful information.

In summary, the paper reveals interesting new data about PAK4. The finding of an inhibitory domain for PAK4 is certainly novel and makes an important contribution to the field, and the finding of splice variants is interesting. The data seems somewhat preliminary in its current form however, and the paper should be better organized.

Referee #2:

The manuscript by Manser and colleagues seeks to clarify the activation mechanism of PAK4 by Cdc42. The experiments that are presented are convincing and the topic is generally of significant interest. However, in it's present form this manuscript does not adequately demonstrate the proposed mechanism and is not acceptable at this time.

The manuscript begins by describing novel splice variants of PAK4, named PAK4b and PAK4c. However, in Figure 1C the regulation of these isoforms by Cdc42 is not presented. For completeness, this should be demonstrated. As well, co-transfection of Cdc42 seems to induce a very subtle increase in GST-Raf13p phosphorylation when comparing lanes 1 and 2. These bands should be quantified or a more sensitive method utilized to clearly bolster the authors' arguments. As well, the label on Figure 1B should be corrected to read "PAK4b" and "PAK4c". Do the PAK4-S474 phosphorylation levels differ between isoforms in the cell lines examined by immunoblotting?

The deletion mutants that are introduced in Figure 2 are helpful to identify the broad region of the PAK4 AID, however missense mutagenesis of key residues predicted to be important for autoinhibition should be examined in the assays presented to confirm the functionality of this domain. As well, The presented model suggests that PAK4 functions as a monomer. Is this the case in cells? This is important to address experimentally. Could dimerization explain the remaining Ser474 phosphorylation that is observed when kinase-dead PAK4 was exogenously expressed in Figure 1?

Several unsubstantiated statements are made on page 5. How similar are the Cdc42-binding fold structures from PAK1 and PAK4? This can be calculated. Is it accurate to say that PAK groups I / II emerged from the newly categorized group III kinases? Is assessment of the AID sufficient to draw these conclusions, and can the sequence divergence accurately be used to identify common ancestors? This requires a more in-depth analysis to be convincing.

As presented in Figure 4B, the evidence that phosphorylation of Ser474 site is not correlated with kinase activity would be supported by utilizing the reversible PAK4 inhibitor that is described in the text (Murray et al., 2010) and is commercially available.

Referee #3:

General
There has long been controversy as to the nature of class II PAKs activation. Potentially this manuscript provides an answer and would therefore be of major significance. However the presentation and nature of the work make it difficult to interpret. I found this manuscript very difficult to read and to my mind some major explanatory segments seemed to be completely missing from the text. There are phrases used throughout that have an ambiguous quality about them, for example the phrase " ... but the isolated kinase retains no memory of this state". One is left questioning the meaning of the statement rather than considering the significance of the data. There may be an important story worth telling here but it is lost in the muddled and complicated presentation.

There is good evidence that N-terminal regions of PAK4 may act to auto-inhibit full length activity but some of the experiments provided here are lacking important controls and are not readily accessible to scrutiny. Moreover, the evidence for constitutive S474 phosphorylation is not as compelling as the authors would lead us to believe. Likewise, the role of Cdc42 binding is not consistently investigated. The only evidence presented for Cdc42 regulation is via an indirect IP, but in other parts of the manuscript the authors return to conventional PAK4 IPs to rule out Cdc42 involvement; even though they have disputed its validity. The cellular validation of constitutive phosphorylation is particularly poor, and the authors have not made any attempt to address the recent finding that PKD can phosphorylate PAK4 at serine S474. Taking all these weaknesses into

consideration I do not consider this manuscript acceptable for publication in EMBO Reports. I also think that this kind of data is not best represented in a report format where you have to have a very clear story and be really succinct in your results/discussion. I would suggest re-submission of a modified manuscript to a journal where it could appear as a full article.

Detail
Figure 1.
A. Alternatively spliced form of PAK4 have been previously reported in the database. How does the isoform reported here differ, or are they the same?
B. Where is the evidence from the authors laboratory for the existence of these isoforms apart from smaller bands on a western blot? The authors allude to mRNA and an alternative splice of exon 4. However there is no supporting evidence that I can see.
C. Did the authors try to repeat this blot with a second PAK4 antibody to see if the bands they detect are specific to PAK4? Most antisera available are not PAK4 specific, picking up PAK6 is common. Where is PAK4c in the whole cell lysates?
D. Text Top of page 4 (The smaller alternate spliced PAK4 isoforms (i.e. PAK4b or c) showed negligible activity (lanes 3 and 4) relative to the catalytic domain PAK4 (Cat), whose activity is similar to the full-length PAK4(S445N) mutant (Gnesutta et al, 2001).
Does this text refer to figure 1C?
E. Why have the authors used GST-Raf13p as the substrate instead of histone or MBP - an explanation is lacking. It makes this result very hard to compare with previously published literature. Is the affinity for GST-Raf13p the same higher/lower than histone /MBP?
Although it is true that WT PAK4 autophosphorylation levels can be low in a kinase assay, it is highly unusual to see no signal at all in lane 1 and 2. How do the authors account for this?
Why does PAK4a-CA autophosphorylate if the authors are proposing that the PAK4 is already constitutively phosphorylated on serine 474 in their assay? PAK41b has little kinase activity - does it bind substrate with equal efficiency?

Figure 2.
A. Where is PAK5 in the line -up?
B. It is not clear to me what the authors think the cause of S474 phosphorylation is. Are they saying that PAK4 auto-phosphorylates itself immediately and in an unregulated manner so that when it is purified its already phosphorylated on S474? Or are they inferring that S474 phosphorylation has an alternative origin? Why is PAK4-kinase dead not phosphorylated on S474 if it is not an autophosphorylation site?
C. The authors do not take into account the recent report that protein kinase D (PKD) phosphorylates PAK4 at serine 474 (Protein kinase D regulates cofilin activity through p21-activated kinase 4. Spratley SJ, Bastea LI, Döppler H, Mizuno K, Storz P. J Biol Chem. 2011; 286:34254-61).
How does this finding of a specific kinase phosphorylation event relate to their studies?

Figure 3
A. In the FlagIP lane 2 and 3 do the authors know if any other PAK/related kinases pull down with Cdc42? One would certainly expect to see PAK1.
B. If they have been excluded from the assay, how was this done?
C. If the interaction between PAK4 and Cdc42 is labile how is it maintained sufficiently to increase kinase activity in lane 2 and 3?
D. What is the serine 474 phosphorylation status of the PAK4 used in this assay?
E. What is the S41 status of the PAK IP'd in Figure 3A?

Figure 4
A. The figure is not at all clear - how are the authors ruling out a correlation between S41 and activity if the cat domain doesn't contain an S41 site and they have already shown that in their assay Cdc42-stimulated PAK4 activity requires IP of Cdc42 not PAK4? Cross reference here to my comment on Figure 3E.
B. The LnCap cells used here were only serum starved for 90 mins. This is hardly sufficient time to allow for the effects of serum depletion on cell metabolism.
I would like to see this experiment repeated in an HGF responsive cell line - where it has previously been reported by more than one group that the S474 signal and PAK4 kinase activity are correlated

with decreased levels in serum starvation and elevated levels in HGF-stimulated cells. I would also like to see quantification across three independent experiments. The S474 signal is very poor in these cells given that this is an IP. - it is very hard to judge these levels and it looks like S474 goes down +10%serum. Quantification definitely required here.
C. Why was sorbitol chosen as the stimulant?
D. The evidence for a Cdc42-mediated change in activity is very weak

Suppl. Figures

Figure S1
I don't see the point of comparing The PAK1 AID with that of WASP: WASP isn't even a kinase as far as I am aware.

Figure S2 -
B. Is this a true measure of AID activity or rather the kinase activity? Just because those point mutations don't increase activity doesn't mean the helix is not involved in AID (if it exits) binding. Why does WT PAK4 now suddenly have an autophosphorylation signal?

Figure S3
A. There is no quantification or input blot for levels of Flag-PAK4 IP
B. Surely if E.coli purified PAK4 has S474 phosphorylation it MUST be an autophosphorylation site?

Examples of PAK4 S474 levels increasing following stimulation.
• Nekrasova and Minden 2011
• Journal cellular physiology HaCaT cells +KGF or +UVB or +KGF+UVB 637-
• Mol. Cell Biol Paliouras et al., 2009 following met and gab overexpression S474 level increases MDCK cells
• JBC Li et al., 2010 figure 7 increased S474 phosphorylation with B5

None of these reports is discussed.

Examples of WT autophosphorylation
• HA-PAK4 in NIH 3T3 Cells EMBO Abo et al., 1998
• HA-PAK4 in MDCK cells JCS Wells et al., 2002
• Flag-PAK4 in Li et al., 2010 JBC fig 5 and 6
• Flag-PAK4 autophosphorylation of FL which increases with time on VN in Li et al., 2010 MBC

No critical evaluation provided by the authors

Resubmission - authors' response 06 March 2012

Thank you for the opportunity to resubmit our manuscript. It is clear the reviewers consider this an important piece of work that is of general interest. As they indicate "The experiments that are presented are convincing and the topic is generally of significant interest." Reviewer 3 wanted more evidence to overturn the substantial body of literature that is at odds with our model (which we have now done). I suspect the request to cite a long list of papers provided by the reviewer reveals a clear conflict of interest (since journals discourage requests for self citation). I'm sure you can deal with this appropriately.

As ever, additional experiments are requested to make a stronger case. This has involved replacing about 1/2 of the panels. The paper has been re-written to improve the 'readability' of the manuscript (as requested by reviewer 1).

The key issues for all the reviewers is to convincingly show PAK4 is constitutively phosphorylated on the A-loop Ser-474 (controversial given the prevalence of this

modification as a marker of 'kinase activity') and demonstrate the PAK4b isoform *in vivo*. Both of these are addressed by developing completely new anti-PAK4 & anti-pS474 antibodies - with much improved characteristics. A direct measure of activation loop status of endogenous PAK4 in cell lysates confirms the over-expression data. In all cases the activity status (or cell treatment) is independent of pS474 modification. This new western data (Figs 1C and 4B) is validated using PAK4 siRNA (Fig. 1C).

The new figures relating to those parts of the manuscript that the reviewers felt needed revising have been updated as follows:
Figure 1 Parts A, B(new), C(new), D(new).
Figure 2 Parts A and D(new).
Figure 3 Parts A(new) and C(new).
Figure 4 Parts B(new), C(new).

Thus our revised paper provides strong evidence for PAK4 being constitutively phosphorylated on it's A-loop, being inhibited by an AID related to PAK1, and being activated directly by Cdc42.
All these findings are unexpected and informative.

Based on these many changes, and the enthusiasm shown by reviewers 1 and 2 we believe the paper suitable for publication in EMBO R. The..pion-by-point rebuttal is given below.

Point-by-Point Response to Reviewers:

Referee #1:
We are pleased that the referee is enthusiastic about our paper and considers that "The finding of an inhibitory domain for PAK4 is certainly novel and makes an important contribution to the field, and the finding of splice variants is interesting." We agree with the reviewer that there is a need to clarify aspects of the paper to improve the readability of the manuscript. This has now been done and we have removed some of the superfluous material such as Ser41 modification (the new text with changes marked up is included for reference).

The key issue of whether PAK4 is indeed constitutively phosphorylated at Ser-474 is resolved since we have developed a new anti-pS474 antibody that allows us to test activation loop status in cell lysates with confidence. Comparisons are provided in Fig. S1 of these new antibodies versus those from Cell Signaling. This new Western data (cf. Figs 1C and 4B) validated by PAK4 siRNA, demonstrates that PAK4 in mammalian cells is phosphorylated on Ser474 (Figs 1C and 4B), even when Cdc42 is removed. We provide confirmatory RT-PCR data in support of the existence of an alternate spliced PAK4b. During this re-evaluation we found that mouse cells do not produce PAK4b, which provides an additional control. Thus our revised paper provides strong evidence for PAK4 being constitutively phosphorylated on it's A-loop, being inhibited by an AID related to PAK1, and being activated directly by Cdc42.

In this paper the authors present interesting new findings about the protein kinase PAK4. Specifically they identify new splice variants of PAK4, which have internal deletions, they find that PAK4 contains an auto inhibitory domain, and they find that the activation loop Ser474 is constitutively phosphorylated and not regulated by external factors, and finally they find that binding of Cd42 activates PAK4 but that the isolated PAK4 has no memory of this state. These are all interesting and novel findings, but do not always seem to fit together into one coherent story. The paper could be better organized, and there is some data does not seem to add much to the main theme of the paper. Furthermore, the paper should be edited for writing and grammar, so that it reads more clearly, and this could alleviate some of the problems described above. Some specific comments are below.

1. In the Introduction section the authors discuss the role for PAK4 in cancer. PAK4 also has a number of other important functions, however, including roles in development.
We now include information in the introduction relating to the role of PAK4 in development as requested.

2. In the first paragraph of the results section the sentence "Human PAK4 (591 residues) is a ubiquitous kinases; it transpires a mRNA encoding a smaller PAK4 is relatively abundant" does not make sense. This sentence should be clarified and the entire manuscript should be carefully edited.
The new version of the manuscript has been re-written as requested. In particular the new data in Fig. 1b indicates that PAK4 is alternate spliced in human but not mouse cells. This is accompanied by new Western data including PAK4 siRNA knockdown to confirm the identity of the various bands (Fig. 1C).

3. Figure 1B. The finding of a shorter PAK4a transcript is interesting and supported by genome sequencing. However, the data in the western is not entirely clear. Flag tagged full length PAK4 (PAK4a) is bigger than endogenous PAK4 as is expected, but flag tagged PAK4b seems to be smaller than the indicated endogenous protein. Why is this? And why is the band in the B16 lane smaller than the one in the N1E lane. Is this PAK4c? Also, the flag tagged and endogenous proteins should be run on the same gel here, for size comparisons.
We agree that the original Western data in Fig. 1 was ambiguous. The revised figure uses a (new) more sensitive anti-PAK4 antibody which shows the presence of PAK4a and PAK4b which are both knocked down by SiRNA targeting the RNA sequence of kinase domain. The size problems noted by the reviewer probably relate to other publications using these Cell Signaling antibodies : there are multiple non-specific bands depending on cell lines (see Fig. S1). Our new anti-PAK4 by contrast recognizes PAK4b in extracts from human but not mouse (B16) cells. The low levels of PAK4b suggest this as a minor isoform, while PAK4c is not represented in the human EST database.

The finding of the internal deletion mutant is interesting, but does not seem to lend strong support for the presence of an auto-inhibitory domain, and does not really seem to add much to the paper.
The existence of a smaller PAK4b represents a 'natural' isoform of the kinase that containing only 68 residues of conserved N-terminal regulatory sequence (including NLS, CRIB, AID). Although a minor species as determined by Western blotting (Fig. 1C), the fact that 68 residues are sufficient for inhibition (as amply demonstrated for our synthetic minimal PAK4s) is highly significant. The other ~250 non-catalytic amino-acids are apparently not needed for auto-inhibition. We have spelt this out with respect to identification of the minimal AID.

4. **Figure 1C**. Why does the catalytically active PAK4 have strong activity even though it has the later described auto inhibitory domain?
The widely used full-length active PAK4(S445N) mutant alters the ATP binding pocket (to resemble PAK1), and perhaps disrupts the AID interaction. No rationale for the mode of action of this mutation is provided by those who have solved the PAK4 structure. The PAK4(S445N) exhibits an activity somewhat higher than that of the catalytic domain alone (lane 6), suggesting that this mutant is 'inappropriately' active. This is now mentioned (p4 para 1).

It would be interesting if the authors also looked at the PAK4(S474E) mutant here. The results could strengthen their argument.
As the reviewer points out full-length 6His-PAK4(S474E) should show low activity, if our model is correct. Indeed the recombinant protein when assayed *in vitro* is not hyper-active (Fig. S3B), nor does it exhibit a typical 'diffuse' mobility like the active PAK4(S445N).

Also in the figure, the addition of Cdc42 seems to lead to a slight increase in Raf13 phosphorylation in response to expression of the full length protein. Why is this? This is not addressed. A small amount of Cdc42 will likely co-purify with the immuno-precipitated PAK4 leading to its activation (this is now mentioned p4, para1).

5. In **Figure 2B** as well as 1C, the authors look at p32 labelling as an indication of auto-phosphorylation. However, it is not clear which sites are being phosphorylated. More discussion or speculation about this would be helpful. Also in 2C, the delta50PAK4b seems to have a low auto-phosphorylation level but a high level of Raf phosphorylation. How can this be explained? This should be addressed.
We now comment that residues 68-202 (exon 4) likely contain PAK4 auto-phosphorylation site(s) and provide a list of sites (identified in high throughput phospho-proteomics) that could be auto-phosphorylation sites (Fig. 2D).

6. **Figure 4B.** This figure was the most troubling. The authors show that S474 phosphorylation does not correlate with treatment with a number of stimuli including serum and sorbitol. However, how do we know that those are even activators of PAK4? The positive control is p38, but that does not mean that PAK4 is activated by these stimuli. Without another way to look at PAK4 activation, these experiments area not that useful. What about looking at PAK4 auto-phosphorylation here? Furthermore, 90 minute serum starvation is probably not sufficient for examining serum stimulation, especially in a tumor cell. Why was only once cell type chosen for these experiments? Regarding the timing of serum stimulation, some pathways may be activated quite transiently, within 1 - 2 minutes, so that a more detailed time course could also more useful information.
We agree with the reviewer that the analysis of PAK4 in cells is problematic particularly as the commercial anti-pS474 detect multiple bands depending on cell line (Fig. S1). Our new anti-pS474 PAK4 antibody can detect endogenous pS474 in various cell lines (new Fig. 1C and 4C). This removes the requirement to analyse immuno-precipitated proteins, and avoids any *in vitro* incubation. At the request of another reviewer we tested PAK4 status in HeLa cells after o/n starvation and HGF treatment at times points of maximal MAPK activation (Fig. 4B). No significant change in pS474 status is noted. This fits our data regarding the level of pSer474 comparing immuno-precipitated and recombinant PAK4 (Fig 4C).

In summary, the paper reveals interesting new data about PAK4. The finding of an inhibitory domain for PAK4 is certainly novel and makes an important contribution to the field, and the finding of splice variants is interesting. The data seems somewhat preliminary in its current form however, and the paper should be better organized.

Referee #2:
We thank the reviewer for positive comments on out work which as the reviewer point out is in general "convincing". Based on the feedback from the reviewer we have sought to improve the data which is reflected in the completely new data presented in Fig 1B, C; Fig 3A and Fig. 4B.

The manuscript by Manser and colleagues seeks to clarify the activation mechanism of PAK4 by Cdc42. The experiments that are presented are convincing and the topic is generally of significant interest. However, in it's present form this manuscript does not adequately demonstrate the proposed mechanism and is not acceptable at this time.

The manuscript begins by describing novel splice variants of PAK4, named PAK4b and PAK4c. However, in Figure 1C the regulation of these isoforms by Cdc42 is not presented. For completeness, this should be demonstrated. As well, co-transfection of Cdc42 seems to induce a very subtle increase in GST-Raf13p phosphorylation when comparing lanes 1 and 2. These bands should be quantified or a more sensitive method utilized to clearly bolster the authors' arguments. As well, the label on Figure 1B should be corrected to read "PAK4b" and "PAK4c". Do the PAK4-S474 phosphorylation levels differ between isoforms in the cell lines examined by immunoblotting?
In the new version of the manuscript we have investigated PAK4 isoforms in 3 cell lines (new Fig. 1B/C). Using a new antibody directed to PAK4, the smaller PAK4b is detected in human but not mouse cell lines. This matches the data from RT-PCR analysis using primers

that span exon 4 (Fig. 1B). These two isoforms of human PAK4 react with antibodies to pS474, and both signals PAK4a /PAK4b bands are knocked down by an siRNA directed to the PAK4 catalytic domain (Fig. 1C). With respect to the activity of Flag-PAK4 (new Fig 1E) the corrections have been made to this figure according to the reviewers' suggestions. The cell lines in Fig. 1C are tested with the anti-pS474 as requested.

The deletion mutants that are introduced in Figure 2 are helpful to identify the broad region of the PAK4 AID, however missense mutagenesis of key residues predicted to be important for auto-inhibition should be examined in the assays presented to confirm the functionality of this domain.
The existence of a smaller PAK4b represents a 'natural' isoform of the kinase that is auto-inhibited via N-terminal 68 residues of regulatory sequences (encompassing NLS, CRIB , AID). Importantly when these 68 residues are attached to the kinase domain (PAK4s) the PAK4 is fully inhibited (Fig. 2B).

The detailed mutagenesis of the PAK4 AID is being performed in conjunction with the NMR determination of the AID: PAK4 cat structure. This will give a clear picture of how the AID operates, but is beyond the scope of this paper.

As well, the presented model suggests that PAK4 functions as a monomer. Is this the case in cells? This is important to address experimentally. Could dimerization explain the remaining Ser474 phosphorylation that is observed when kinase-dead PAK4 was exogenously expressed in Figure 1?
Gel filtration of PAK1 versus PAK4 suggests the latter is a monomer (this is mentioned as unpublished data on p9) the will be presented elsewhere with respect to the contrasting oligomeric state of PAK5. It is certain that PAK4 can undergo auto-phosphorylation in *E.coli* . As the reviewer points out low level phosphorylation of kinase-inactive PAK4 by endogenous PAK4 is likely..

Several unsubstantiated statements are made on page 5. How similar are the Cdc42-binding fold structures from PAK1 and PAK4? This can be calculated. Is it accurate to say that PAK groups I / II emerged from the newly categorized group III kinases? Is assessment of the AID sufficient to draw these conclusions, and can the sequence divergence accurately be used to identify common ancestors? This requires a more in-depth analysis to be convincing.
We have expanded our comments on the structure of the PAK1 and PAK4 CRIB/AID (p 5, para 2) cf. "Both PAK1 and PAK4 CRIB sequences forms an almost identical Cdc42 binding fold (pdb: 1E0A and 2OV2). In these structures the N-termini of the PAK4 and PAK1 CRIBs bind as a b-sheet to Cdc42 strand b2, and similar CRIB residues bind to switches I and II of Cdc42.GTP. The putative helix 1 of the PAK4 AID (marked on Fig. 2A) is indeed an a-helix when bound to Cdc42". We have revised down our comments about the evolutionary relationship between the protozoan PAKs and the group I and group II kinases. This probably requires additional experiments to be performed with protozoan PAKs. The new text states "Given that the AID is fundamental to kinase regulation it seems likely that both group I and group II AIDs originate from a simpler protozoan PAK module."

As presented in Figure 4B, the evidence that phosphorylation of Ser474 site is not correlated with kinase activity would be supported by utilizing the reversible PAK4 inhibitor that is described in the text (Murray et al., 2010) and is commercially available.
We have now provided data in Figure S1B that treatment of cells with a generic kinase inhibitor staurosporine, or a phosphatase inhibitor calyculin has no effect on the pS474 signal detected in U2OS cells. These experiments argue that there is little turnover of PAK4 pS474 under standard cell culture conditions. Thus modification of S474 is not dynamic & likely occurs shortly after synthesis (as for PKA). Further if our model is correct full-length 6His-PAK4(S474E) should also be inactive (ie inhibited). Indeed the recombinant protein when assayed *in vitro* is not hyper-active (Fig. S3B), nor does it exhibit a typical 'diffuse' mobility characteristic of the active PAK4(S445N).

Referee #3:
We agree with the reviewer that "There has long been controversy as to the nature of class II PAKs activation. Potentially this manuscript provides an answer and would therefore be of major significance. The paper has been completely re-written to make the story more coherent.

That PAK4 activation loop phosphorylation is not used to regulate kinase activity is unusual but not unique. In the revised version of this manuscript we provide strong evidence using a sensitive new anti-pS474 antibody. Comparisons are provided in Fig. S1 of these new antibodies versus those used previously. This new western data (cf. Figs 1C and 4B), is validated by PAK4 siRNA. We provide confirmatory RT-PCR data in support of an alternate spliced PAK4b in human cell lines. Mouse cells do not produce the smaller PAK4b, which provides an important additional control. Thus our revised paper goes well beyond the original in demonstrating that PAK4 is constitutively phosphorylated on it's A-loop, is inhibited by an AID that is located between residues 20-68, and directly activated by Cdc42. We do not feel there is ambiguity in these results, and the text has been extensively changes to reflect these three key findings (as requested).

General
There has long been controversy as to the nature of class II PAKs activation. Potentially this manuscript provides an answer and would therefore be of major significance. However the presentation and nature of the work make it difficult to interpret. I found this manuscript very difficult to read and to my mind some major explanatory segments seemed to be completely missing from the text. There are phrases used throughout that have an ambiguous quality about them, for example the phrase " ... but the isolated kinase retains no memory of this state". One is left questioning the meaning of the statement rather than considering the significance of the data. There may be an important story worth telling here but it is lost in the muddled and complicated presentation.

There is good evidence that N-terminal regions of PAK4 may act to auto-inhibit full length activity but some of the experiments provided here are lacking important controls and are not readily accessible to scrutiny. Moreover, the evidence for constitutive S474 phosphorylation is not as compelling as the authors would lead us to believe. Likewise, the role of Cdc42 binding is not consistently investigated. The only evidence presented for Cdc42 regulation is via an indirect IP, but in other parts of the manuscript the authors return to conventional PAK4 IPs to rule out Cdc42 involvement; even though they have disputed its validity. The cellular validation of constitutive phosphorylation is particularly poor, and the authors have not made any attempt to address the recent finding that PKD can phosphorylate PAK4 at serine S474.

Comments.
We agree that the original requirement to immunoprecipitate endogenous PAK4 in order to analyse the pS474 status was less than ideal (the Cell Signaling anti-pPAK4 is particularly weak (Fig. S1). Using our new anti-pS474 we have now conducted the HGF studies as requested (Fig. 4B). Further exposing cells to generic phosphatase or kinase inhibitors does not alter the pS474 status (Fig. S1B).

This data is backed up by extensive experiments using mutant PAK4 constructs which all points to the kinase being phosphorylated at pS474 regardless of activity status (Figs 1 and 2). The additional controls requested above have been added. In summary new panels relating to those parts of the manuscript that the reviewer felt needed revising are updated as follows:

Figure 1 Parts A, B(new), C(new), D(new).
Figure 2 Parts A and D(new).
Figure 3 Parts A(new) and C(new).
Figure 4 Parts B (new), C(new).

Although Cdc42 clearly binds PAK4 the percentage of complex that survives a typical PAK4 IP protocol has never been tested. If it were say ~10% then the increase in overall activity would

be 50% (based on our 5-fold stimulation) which would be close to the error of measuring PAK4 by Western blotting. With respect to the involvement of PKD in PAK4 modification at serine S474 , inspection of the data in the paper suggests the authors were not actually assaying PAK4 (see detailed comments below). A full point-by-point rebuttal is given below (in blue).

Figure 1.

A. Alternatively spliced form of PAK4 have been previously reported in the database. How does the isoform reported here differ, or are they the same?
The PAK4b reported here has not been described in the literature to our knowledge although widely represented in the public databases. The new data in Fig. 1B/C confirms the smaller mRNA, and the protein at lower abundance. This isoform is not found in mouse EST databases.

B. Where is the evidence from the authors laboratory for the existence of these isoforms apart from smaller bands on a western blot? The authors allude to mRNA and an alternative splice of exon 4. However there is no supporting evidence that I can see.
As requested the information is now provided in Figs 1B ad 1C.

C. Did the authors try to repeat this blot with a second PAK4 antibody to see if the bands they detect are specific to PAK4? Most antisera available are not PAK4 specific, picking up PAK6 is common. Where is PAK4c in the whole cell lysates?
Our new anti-PAK4 antibody is highly specific to PAK4 unlike the antibody we used previously (ie old Fig.1B). The data we provide here matches RT-PCR to Westerns.

D. Text Top of page 4 (The smaller alternate spliced PAK4 isoforms (i.e. PAK4b or c) showed negligible activity (lanes 3 and 4) relative to the catalytic domain PAK4 (Cat), whose activity is similar to the full-length PAK4(S445N) mutant (Gnesutta et al, 2001).
Does this text refer to figure 1C?
The text here includes a full description of the inhibited forms.

E. Why have the authors used GST-Raf13p as the substrate instead of histone or MBP - an explanation is lacking. It makes this result very hard to compare with previously published literature. Is the affinity for GST-Raf13p the same higher/lower than histone /MBP?
Such a Raf13 peptide was used to biochemically purify the 'Raf1 S338/9' kinase (PAK2). Similarly PAK4 can activate Raf1 (Cammarano et al.,2005 *Mol Cell Biol.* 25:9532-42) In Fig. S3 we compare the behaviour of GST-Raf13 and full-length Bad substrates, showing that both PAK4 and PAK1 kinases can phosphorylate these; PAK4 is actually a better Raf1 kinase. We are wary of using very basic substrates such as MBP and histone because it is known that PAK2 is activated by such proteins *in vitro* (Jakobi et al., 2000 Eur J Biochem. 267: 4414-21).

Although it is true that WT PAK4 auto-phosphorylation levels can be low in a kinase assay, it is highly unusual to see no signal at all in lane 1 and 2. How do the authors account for this?
The exposure times for the assay are based on the signal of the constitutively active forms. Raf1 peptide does bias assays towards PAKs rather than contaminating kinases. MBP and histone are good as a generic substrate for basic-directed kinase, but as a result give high backgrounds.

Why does PAK4a-CA autophosphorylate if the authors are proposing that the PAK4 is already constitutively phosphorylated on serine 474 in their assay? PAK41b has little kinase activity - does it bind substrate with equal efficiency?
PAK4a-CA is the full-length kinase that can undergo auto-phosphorylation on sites other than Ser474 (ie in the N-terminal region). *In vivo* sites of PAK4 modification based on the Netphos phospho-proteomic database, and thus potential auto-phosphorylation sites are given in Fig. 2D.

Figure 2.

A. Where is PAK5 in the line -up?
We have added human PAK5 as requested.

B. It is not clear to me what the authors think the cause of S474 phosphorylation is. Are they saying that PAK4 auto-phosphorylates itself immediately and in an unregulated manner so that when it is purified its already phosphorylated on S474? Or are they inferring that S474 phosphorylation has an alternative origin? Why is PAK4-kinase dead not phosphorylated on S474 if it is not an autophosphorylation site?
We suggest that PAK4 is auto-phosphorylated at S474 - probably shortly after synthesis (see new text). This fits with the idea that recombinant PAK4 is also phosphorylated on S474. These issues are more clearly exposed in the new version.

C. The authors do not take into account the recent report that protein kinase D (PKD) phosphorylates PAK4 at serine 474 (Protein kinase D regulates cofilin activity through p21-activated kinase 4. Spratley SJ, Bastea LI, Döppler H, Mizuno K, Storz P. J Biol Chem. 2011; 286:34254-61). How does this finding of a specific kinase phosphorylation event relate to their studies?
We have looked at the data in the Spratley, paper in detail. The authors show that inactive PAK4.K350M can be phosphorylated by PKD1.CA on the activation loop (if the kinase were WT it would be already modified, according to our data). The key panel relates to the detection of pS474 on endogenous PAK4 in cells transfected with active PKD (using the Cell Signaling anti-pS474). The PKD1.CA, PKD2.CA and PKD3.CA expression give several-fold increase in signal of a band of ~50 kDa (ie definitely the wrong size). This indicates a target other than PAK4. Referencing this paper is likely to confuse the readership.

Figure 3.
A. In the Flag IP lane 2 and 3 do the authors know if any other PAK/related kinases pull down with Cdc42? One would certainly expect to see PAK1.
B. If they have been excluded from the assay, how was this done?
The background kinase activity in the Cdc42 IP (see new Fig 3a) may on the face of it be strange. But in such over-expression experiments the PAK4 levels are probably >50 times that of endogenous kinase (based on Fig. 4C), so the low activity here is reasonable given that even Pak1 has poor reactivity to the Raf1 peptide (Fig S3B).

C. If the interaction between PAK4 and Cdc42 is labile how is it maintained sufficiently to increase kinase activity in lane 2 and 3?
We are using an IP buffer containing magnesium (see methods) which attempts to maintain the PAK4 : Cdc42 complex. If PAK4 dissociates it can be 'recaptured' by excess Cdc42 in the Sepharose.

D. What is the serine 474 phosphorylation status of the PAK4 used in this assay?
E. What is the S41 status of the PAK IP'd in Figure 3A?
The pS474 status of the kinase in this assay is now provided. Because we do not see significant S41 phosphorylation with PAK4+ Cdc42, this data has been removed.

Figure 4.
A. The figure is not at all clear - how are the authors ruling out a correlation between S41 and activity if the cat domain doesn't contain an S41 site and they have already shown that in their assay Cdc42-stimulated PAK4 activity requires IP of Cdc42 not PAK4? Cross reference here to my comment on Figure 3E.
We agree the pS41 data is difficult to reconcile with the mechanism proposed, and has been removed (see comment above). It is clear that the PAK4(S445N) mutant undergoes extensive auto-phosphorylation that is not seen for PAK4 + Cdc42 or PAK4(S474E) as shown in Fig. S3. Perhaps because the mutation makes the kinase behaves like active PAK1?

B. The LnCap cells used here were only serum starved for 90 mins. This is hardly sufficient time to allow for the effects of serum depletion on cell metabolism. I would like to see this experiment repeated in an HGF responsive cell line - where it has previously been reported by more than one group that the S474 signal and PAK4 kinase activity are correlated with decreased levels in serum starvation and elevated levels in HGF-stimulated cells. I would also like to see quantification across three independent experiments. The S474 signal is very poor

in these cells given that this is an IP. - it is very hard to judge these levels and it looks like S474 goes down +10% serum. Quantification definitely required here.
C. Why was sorbitol chosen as the stimulant?
We agree that the original data using the IP/anti-pS474 was unsatisfactory. Thus the use of a new anti-pS474 Ab is important. We have used HGF treatment of Hela cells after over-night starvation (as suggested) and confirm HGF is activating ERK as expected. The pPAK4 signal is unambiguously detected in cell lysates : there is no indication of a change in pS474 signal under this regime.

D. The evidence for a Cdc42-mediated change in activity is very weak
We have repeated the experiment several times (3) as suggested (Fig. 3A) and quantified the combined results (Fig 3C).

Suppl. Figures
Figure S1 I don't see the point of comparing The PAK1 AID with that of WASP: WASP isn't even a kinase as far as I am aware. Discussion of WASP is removed.

Figure S2B. Is this a true measure of AID activity or rather the kinase activity? Just because those point mutations don't increase activity doesn't mean the helix is not involved in AID (if it exits) binding. Why does WT PAK4 now suddenly have an auto-phosphorylation signal?
We would assume that AID interaction with the kinase domain does underlie its action. Mutations to either the kinase or AID (which disrupt this interaction) will lead to kinase activation. The exposure time is long enough to detect WT auto-phosphorylation.

Figure S3 A. There is no quantification or input blot for levels of Flag-PAK4 IP
The amount of PAK4 in this assay is visible in Coomassie stained blot.

B. Surely if E.coli purified PAK4 has S474 phosphorylation it MUST be an autophosphorylation site?
We agree that PAK S474 auto-phosphorylation is most likely & PKD is an unlikely upstream kinase.

Examples of PAK4 S474 levels increasing following stimulation.
• Nekrasova and Minden 2011
• Journal cellular physiology HaCaT cells +KGF or +UVB or +KGF+UVB 637-
• Mol. Cell Biol Paliouras et al., 2009 following met and gab overexpression S474 level increases MDCK cells
• JBC Li et al., 2010 figure 7 increased S474 phosphorylation with B5
None of these reports is discussed.

Examples of WT autophosphorylation
• HA-PAK4 in NIH 3T3 Cells EMBO Abo et al., 1998
• HA-PAK4 in MDCK cells JCS Wells et al., 2002
• Flag-PAK4 in Li et al., 2010 JBC fig 5 and 6
• Flag-PAK4 autophosphorylation of FL which increases with time on VN in Li et al., 2010 MBC

No critical evaluation provided by the authors

Given that our work fundamentally disagrees with the notion of pS474 as a surrogate of PAK4 activity we have been circumspect about re-evaluating all the data provided in these publications. Since there is longstanding data linking PAK4 activity to HGF-induced motility, we have restricted our experiment to this system.

As reviewer points out, the relative level of PAK4 auto-phosphorylation (in our assays) is much lower than for active forms of PAK4 which is in line with in vitro kinase assays performed with other kinases.

Editor's correspondence 07 March 2012

I have now received the uninvited resubmission of your study on the mechanism of PAK4 activation. After reading it and your point-by-point response, I have decided to have it peer-reviewed once again. Please note that I cannot guarantee the referees will be the same, as this will depend on their availability (especially as this is considered a new submission, rather than a revision). New experts might be asked to assess it if necessary.

I would like to point out that you seem to have misjudged the level of enthusiasm of referees 1 and 2. Both of them considered the study "might be published in EMBO reports", as opposed to the more supportive "should be published" and referee 2 rated the novelty and general interest of the findings as "medium" in the summary evaluation sheet. Nevertheless, they will now have the opportunity to reassess the work if they choose to do so.

I would also like to note that referee 3 did not ask to cite his/her articles, as you implied in your cover letter, and thus unlikely has a conflict of interest. Rather, s/he seems to have brought up very pertinent points, as you frequently acknowledge in your point-by-point response. In this regard, if the outcome of peer-review is positive, we will require that you cite and discuss the studies that disagree with your results, which far from confusing the reader we believe will help to clarify the field.

I will be back in touch as soon as I have a full set of reports.

Yours sincerely,

Editor
EMBO reports

2nd Editorial Decision 28 March 2012

Thank you for your resubmission to EMBO reports. We have now received the three enclosed reports on it. Referees 1 and 2 of the previous version are current referees 1 and 3, and former referee 3 was unavailable, so referee 2 has seen the work anew. As you will see, although referees 1 and 3 are now supportive of publication, referee 2 brings up numerous concerns, some of which were supported by referee 3 upon further discussion.

Given the nature of some of these concerns, they would need to be addressed before publication can be considered in EMBO reports. Of special importance would be to provide the requested validation of the new serine474 antibody and use it in figure 2 (and maybe 4B). As I mentioned in our earlier correspondence and is brought up by this referee, we require that you cite and discuss the previous literature (including the studies that disagree with your results), which will help to clarify the field. Lastly, please go through the whole text carefully and modify the subheadings for clarity, as well as the main text wherever needed. We do appreciate that exploring the contribution of PKD family members to the regulation of serine474 is beyond the scope of this report, but would encourage you to address as many of the other points brought up by referee 2 as possible, as this would undoubtedly strengthen the study, which is essential if it is to change a paradigm in the field.

If the above referee concerns can be adequately addressed, we would be happy to consider your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The paper has been revised, and edited for clarity. The addition of more controls, new antibody staining, and the replacement of several figures, as well as clarification of the text, makes the paper stronger. The paper is now suitable for publication

Referee #2:

The authors present a complex analysis of PAK4 biology. They propose the novel discovery that PAK4 has an autoinhibitory domain (previously this kinase was not thought to contain an AID) and that a serine phosphorylation site in the kinase domain S474 is constitutively phosphorylated. These findings are controversial as the S474 site is used by many researchers as a mark of PAK4 activity. Moreover, the authors present new data indicating that Cdc42 can regulate the kinases activity of PAK4, again a finding that contrasts with previous work suggesting binding of Cdc42 does not elevate PAK4 activity. Finally they report the discovery of a second PAK4 isoform in human but not mouse cells. Given the importance of the findings presented here, especially regarding S474 phosphorylation, the work has to be particularly rigorous. The S474 data relies on the use of a novel unpublished antibody and the authors do not validate this antibody as phospho-specific. Nor do they test whether is recognises other members of the Group II family. Moreover, the discussion of the role of S474 phosphorylation is not detailed enough and there is no attempt to reconcile their results with other work showing this site is regulated by PKD (Spratley et al., 2011). The manuscript is one of four parts and the work does not attempt to elucidate the relationship between these observations and thus each part although interesting feels incomplete.
Specific Points
1. The finding that there is a second PAK4 isoform is interesting but the authors make no attempt to integrate these findings. PAK4a and PAK4b appear to have relatively similar kinase activity and there is no data on any difference between Cdc42 or substrate affinity.
2. The findings relating to S474 use a new antibody raised by this group. We need to see validation that this new S474 antibody (Figure 1C) is phospho-specific. Is the * non-specific band in fact PAK6? Figure S1B is not the best control. Need to CIP lysates and see if S474 signal from new antibody is removed.
3. Why is there a PAK4c on Figure 1E?
4. Agreed PAK4s result would suggest that residues 1-68 can inhibit activity. What happens if you just add the putative AID separately like PAK1 AID experiments? Why does PAK4s have a stronger S474 signal when the other kinase inactive (KD) has virtually none? Whereas delta50PAK4b has high kinase activity and low S474 signal? These results leave me rather confused.
5. Given that PAK4Cat incorporates [32P], the authors suggest an alternative auto-phosphorylation site (Figure 2D) but do not test this hypothesis -why?
6. The kinase dead has S474 phosphorylation how do the authors account for this...does this happen if expressed in a PAK4 knockdown cell line?
7. Having shown that the new S474 antibody is much more sensitive, the authors then switch to using the CST antibody in Figure 2 - why?
8. In Figure 3A the data would be improved by adding in a PAK4:Cdc42 fusion where the PAK4 is point mutated around H1922L to prevent an interaction with Cdc42

9. How is Figure 3D investigating the role of the AID in substrate affinity. Agreed the difference in binding between WT and any of the mutants looks significant, but this does not directly argue for AID involvement. Rather evidence that the AID can block interaction is required. Have the authors ruled out AID blocking ATP incorporation?
10. The AID residues mutated to yield increased PAK4 kinase activity are interesting but have not been linked to either autophosphorylation or Cdc42 interaction and as such are therefore too preliminary to comment on.
11. In Figure 4A the PAK4aAID* has an autophosphorylation signal, but PAK4aCat has none why is this?
12. Figure 4B: please indicate in the figure legend which antibody was used to detect pS474
13. Figure 4C this experiment is weak. Is PAK4 activated by sorbitol? The robust activation of Cdc42 downstream of soribitol does not necessarily signal via PAK4; given data in Figure 4B is this expt really needed?
14. I would like to see the authors use the PKD inhibitor CID755673 and show with their antibody that pS474 is not modulated in Hela cells, to compare with the Spratley et al., 2011 results.

Overall
Whilst there are some really interesting observations here, that have high significance in the PAK4 field, the paper is complicated by developing four different themes but not bringing any of them to conclusion or finding a relationship between the various aspects of PAK4 biology investigated. This leaves the reader not quite sure what the overall message is. This is not helped by a conflict with nature of a short report. If the authors had more word allowance the work could be better explained/expanded. However, adding some of the controls identified above and also re-writing the paper with clearer sub headings- (some of the sub headings are very misleading i.e. "constructing a full length PAK4".) would greatly improve the mauscript.

Referee #3:

The authors have made significant improvements to the manuscript, including the use of a p-PAK4(S474) antibody and in vitro kinase assays to considerably improve testing the activation loop status of PAK4 and also provide clarity to the immunoblots that were previously somewhat ambiguous. The authors have also revised the text in several places to ensure that the conclusions are consistent with the data that is presented. I accept the authors' comments that some of the requested experiments are more appropriate for follow-up manuscripts, and find that the article in its current form is acceptable for publication.

2nd Revision - authors' response 25 April 2012

Referee #1:
The paper has been revised, and edited for clarity. The addition of more controls, new antibody staining, and the replacement of several figures, as well as clarification of the text, makes the paper stronger. The paper is now suitable for publication.

Referee #3:
The authors have made significant improvements to the manuscript, including the use of a p-PAK4(S474) antibody and in vitro kinase assays to considerably improve testing the activation loop status of PAK4 and also provide clarity to the immunoblots that were previously somewhat ambiguous. The authors have also revised the text in several places to ensure that the conclusions are consistent with the data that is presented. I accept the authors' comments that some of the requested experiments are more appropriate for follow-up manuscripts, and find that the article in its current form is acceptable for publication.

Referee #2:

We thank the reviewer for the care taken to go through our paper. As the reviewer says "Given the importance of the findings presented here, especially regarding S474 phosphorylation, the work has to be particularly rigorous". We feel the new version provides water-tight validation of our model.

Our rebuttal to the various issues is provided below.

The authors present a complex analysis of PAK4 biology. They propose the novel discovery that PAK4 has an autoinhibitory domain (previously this kinase was not thought to contain an AID) and that a serine phosphorylation site in the kinase domain S474 is constitutively phosphorylated. These findings are controversial as the S474 site is used by many researchers as a mark of PAK4 activity. Moreover, the authors present new data indicating that Cdc42 can regulate the kinases activity of PAK4, again a finding that contrasts with previous work suggesting binding of Cdc42 does not elevate PAK4 activity. Finally they report the discovery of a second PAK4 isoform in human but not mouse cells.

Given the importance of the findings presented here, especially regarding S474 phosphorylation, the work has to be particularly rigorous. The S474 data relies on the use of a novel unpublished antibody and the authors do not validate this antibody as phospho-specific. Nor do they test whether is recognises other members of the Group II family. Moreover, the discussion of the role of S474 phosphorylation is not detailed enough and there is no attempt to reconcile their results with other work showing this site is regulated by PKD (Spratley et al., 2011). The manuscript is one of four parts and the work does not attempt to elucidate the relationship between these observations and thus each part although interesting feels incomplete.

The reviewer is right is arguing for better characterisation of the (new) pS474 antibody. As requested we now present additional validation of the anti-pS474 (new Fig 2 and S1); these issues are noted in the text. The experiments relating to over-expressed PAK4 in Figs 1-4 used the less sensitive Cell Signaling (anti-pS474) preparation. Thus our conclusions regarding constitutive phosphorylation at pS474 are based on 2 independent preparations of antibody.

As suggested we have tested PAK4, PAK5, and PAK6 which are equally phosphorylated on residues equivalent to pS474 (new Fig 2B), thus demonstrating that this is a general phenomena across the group II kinase family. The mobility these proteins argues against the p72 'non-specific' band being PAK6.

We have looked at the data in the Spratley, paper in detail. The authors show that inactive PAK4.K350M can be phosphorylated by PKD1.CA on the activation loop (if the kinase were WT it would be already modified, according to our data). The key panel relates to the detection of pS474 on endogenous PAK4 in cells transfected with active PKD (Fig. 5). The PKD1.CA, PKD2.CA and PKD3.CA expression give several-fold increase in signal of a band of ~50 kDa (ie definitely the wrong size for PAK4a). Highlighting these problems with the Spratley paper is likely to confuse the readership. There is no convincing evidence (or in this paper) that PAK4 does not undergo autophosphorylation on Ser474. *E coli* PAK4 is purified in a fully phosphorylated state.

We do not agree that this paper is overly complicated and fragmented, but perhaps the original condensed style gave this impression. In this new version we have more space to elaborate. The paper makes 3 straightforward claims/new concepts:
1. PAK4 being constitutively phosphorylated on it's A-loop.
2. PAK4 being inhibited by an AID related to PAK1.
3. PAK4 being activated directly by Cdc42.

These findings are inter-related ie. Since A-loop phosphorylation is not the mechanism of activation, we identify a PAK4 CRIB/AID that when deleted or mutated gives rise to an active form of the kinase. This AID allows Cdc42 to regulate activity.

Specific Points

1. The finding that there is a second PAK4 isoform is interesting but the authors make no attempt to integrate these findings. PAK4a and PAK4b appear to have relatively similar kinase activity and there is no data on any difference between Cdc42 or substrate affinity.
As requested we have now tested Cdc42 binding of the PAK4a and PAK4b as well as the active mutant. We find no significant difference between the isoforms, but the AID mutant does show reduced binding. This is quite interesting given the positions of the mutated residues (R48,R49), and suggests that the AID / CRIB does form a single domain. This complements the data relating to *Drosophila* Mbt where the CRIB HH/LL mutation can increase kinase activity.

2. The findings relating to S474 use a new antibody raised by this group. We need to see validation that this new S474 antibody (Figure 1C) is phospho-specific. Is the * non-specific band in fact PAK6? Figure S1B is not the best control. Need to CIP lysates and see if S474 signal from new antibody is removed.
We provide better data on the selectivity of our anti- pS474 antibody as requested. (Fig. S1B). One can see that the kinase inactive PAK4a is not recognized at all by this antibody. The * band is not PAK6, at least by its size (new Fig. 2B), and insensitivity to PAK6 SiRNA (data now shown).

3. Why is there a PAK4c on Figure 1E?
This 'isoform' was included because it lacks residues 121-285 and yet is inhibited. As there are no ESTs corresponding to this protein we suspect it's a cDNA artifact.

4. Agreed PAK4s result would suggest that residues 1-68 can inhibit activity. What happens if you just add the putative AID separately like PAK1 AID experiments?
Although we have NMR data of an isolated AID with the kinase domain, the affinity is >10 uM and does not effectively inhibit under *in vitro* conditions.

Why does PAK4s have a stronger S474 signal when the other kinase inactive (KD) has virtually none? Whereas delta50PAK4b has high kinase activity and low S474 signal? These results leave me rather confused.
The designation KD is perhaps confusing - this is K350M (no phospho-transfer activity). The residual pS474 signal seen in the PAK4 KD may involve modification by endogenous PAK4 or other kinase(s). We have simplified this figure. The ability of the PAK4s to undergo auto-phosphorylation (while 286-591 does not), suggests at least one auto-phosphorylation site within residues 1-68. We know this is Ser41 but discussion of this is beyond the scope of the paper. The fact that the kinase domain does not undergo auto-phosphorylation is discussed more clearly in our new version.

5. Given that PAK4Cat incorporates [32P], the authors suggest an alternative auto-phosphorylation site (Figure 2D) but do not test this hypothesis -why?
We have tried to make this clearer in the new version. PAK4Cat(287-591) does not incorporate [32P] because the A-loop is already fully modified (Fig. 2C).

6. The kinase dead has S474 phosphorylation how do the authors account for this...does this happen if expressed in a PAK4 knockdown cell line?
We observe residual modification of the kinase inactive PAK4 K350M. This might occur to a limited extent via other basic directed kinases but likely is irrelevant in the case of WT kinase.

7. Having shown that the new S474 antibody is much more sensitive, the authors then switch to using the CST antibody in Figure 2 - why?
The original work was done using the CS antibody which is fine for over-expressed kinase. The new antibody was developed to accurately address the behaviour of endogenous kinase.

8. In Figure 3A the data would be improved by adding in a PAK4:Cdc42 fusion where the PAK4 is point mutated around H19/22L to prevent an interaction with Cdc42. We agree that this would be an interesting control. However Schneeberger & Raabe (2003) found that Drosophila MBT when mutated to HH/LL is somewhat activated (similar to PAK1). With hindsight this likely reflects an indirect effect on the Mbt AID. This is mentioned in the new version.

9. How is Figure 3D investigating the role of the AID in substrate affinity. Agreed the difference in binding between WT and any of the mutants looks significant, but this does not directly argue for AID involvement. Rather evidence that the AID can block interaction is required. Have the authors ruled out AID blocking ATP incorporation? We agree that the mechanisms of AID action remains ambiguous without structural information as to where exactly the AID binds. GEFH1 binds to the catalytic domain 286-591 sequence - which is more accessible when the AID is removed or mutated. There seems to be no problem with this particular interpretation given the AID binds the catalytic domain.

When we solve the structure of the AID-PAK4-Cat complex these detailed biochemical issues can be sorted out.

10. The AID residues mutated to yield increased PAK4 kinase activity are interesting but have not been linked to either autophosphorylation or Cdc42 interaction and as such are therefore too preliminary to comment on.
We have now tested the interaction between Cdc42 and the PAK4 AID* mutant (new Fig. 4B). Surprisingly (to us) the mutations decreases the interaction with Cdc42V12 (by co-transfection). The mutations themselves are not within the 'CRIB' region, and suggest that the CRIB/AID are folded into a single unit much like PAK1. This is now noted. With regard to the serine /threonine mutants we believe the data stands that excludes these as being involved with PAK4 activation.

11. In Figure 4A the PAK4a AID* has an auto-phosphorylation signal, but PAK4aCat has none why is this?

Referring back to our response to point 4 - PAK4aCat has no other auto-phosphorylation site other than Ser-474 - which is already fully phosphorylated.

12. Figure 4B: please indicate in the figure legend which antibody was used to detect pS474.
This has been done throughout.

13. Figure 4C this experiment is weak. Is PAK4 activated by sorbitol? The robust activation of Cdc42 downstream of sorbitol does not necessarily signal via PAK4; given data in Figure 4B is this expt really needed?
One of the reviewers requested that we test levels of pS474 PAK4 specifically in the presence of HGF, since this is regarded as the most robust signal implicated in 'PAK4-dependent' migration. The sorbitol experiment uses IP and anti-pS474 as an alternate means to assessing potential changes in 'activity' status. This is useful supporting data presented in the supplementary Figure 4.

14. I would like to see the authors use the PKD inhibitor CID755673 and show with their antibody that pS474 is not modulated in Hela cells, to compare with the Spratley et al., 2011 results.
See above for our comments on the Spratley et al., (2011) paper. At best one can agree that the kinase inactive version of PAK4 can be modified under over-expression conditions by PKD1 ,2, 3. The role of PKDs on endogenous PAK pS474 are untested.

Overall
Whilst there are some really interesting observations here, that have high significance in the PAK4 field, the paper is complicated by developing four different themes but not bringing any of them to conclusion or finding a relationship between the various aspects of PAK4 biology investigated. This leaves the reader not quite sure what the overall message is. This is not helped by a conflict with nature of a short report. If the authors had more word allowance the work could be better explained/expanded. However, adding some of the controls identified above and also re-writing the paper with clearer sub headings- (some of the sub headings are very misleading i.e. "constructing a full length PAK4") would greatly improve the manuscript.
The editor has agreed to allow an increased character limit of 28,500 and we have therefore added the details suggested by the reviewer to bring the message more into focus. As suggested the subheadings have been changed and this indeed has improved the manuscript.

3rd Editorial Decision 07 May 2012

Thank you for your patience while your study has been assessed by referee 2 of the previous version. S/he is now supportive of the study and has no further comments, and I am thus very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

I have noticed that the manuscript contains an extremely succinct materials and methods section. Basic materials and methods essential to the understanding of the experiments must be described in the main body of the manuscript and cannot be presented in the supplement, although more detailed explanations necessary to reproduce them may be presented as supplementary information. In addition, please note that it is EMBO reports style to divide the Results & Discussion section into subheadings of a maximum of 50 characters in length (including spaces) and not include sub-subheadings. I must therefore ask you to go through your text one more time to incorporate these changes and email us the final text (which we will upload to your manuscript online), before the study can be sent to our production office.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication.

Yours sincerely,

Editor
EMBO Reports