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Activation of Src and transformation by a RPTPα splice mutant found in human tumors

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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 12 October 2010

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office. I now had the opportunity to read the manuscript carefully, check the related literature and discuss it with some members of our editorial team. I am sorry to say that the outcome of this assessment was not a positive one, as we all had to conclude that we are unable to offer publication.

We certainly appreciate identification of partial mechanistic characterization of novel, patient-associated PTP-alpha mutations. We also realize that these truncations seem to escape quality control mechanisms such as NMD. Molecular analysis of PTPalpha245 reveals transformation potential, heterodimer formation with endogenous PTPalpha with no direct impact on catalytic activity. However, and considering the earlier established framework of phosphotyrosine displacement, heterodimerization seems to abrogate Grb2-binding. Despite appreciating identification/classification of novel, potentially causal disease mutations, we still had to realize that the existing conceptual framework significantly reduces the enthusiasm for the current paper. Therefore and in the absence of definitive, preferably structure-based understanding combined with causal evidence for tumorigenesis from in-vivo assays we were all not convinced that the current submission would provide both the necessary conceptual advance or the amount of definitive novel molecular insight that we have to demand according to the aim and scope of our relatively broad and highly competitive journal. Also in the interest of your and the papers time, we decided to return the manuscript to you to with the message that we are unable to offer further proceedings.

Please let me add that we are looking for complete papers that describe original research of general rather than specialist interest in molecular biology and we can only afford to select those manuscripts that merit urgent publication because they report novel findings of wide biological significance, sufficient level of definitive molecular understanding and tested physiological relevance. This is in fact a very tall order and it means that we end up rejecting by far the majority of the very many manuscripts we receive every day at our editorial office. I am sorry to have to disappoint you on this occasion, but I hope that you nevertheless might consider our journal for publication of your future studies.

Yours sincerely,

Editor
The EMBO Journal

Rebuttal 15 November 2010

Since receiving your negative response to our submission on Oct 12, we have completed in vivo tumorigenicity studies on the splice-mutant PTPalpha245 using a nude mouse xenograft model. These studies show that PTPalpha245 is tumorigenic in vivo (with tumors appearing in 4-8 days in all cases) and, combined with the exceptionally high frequency of occurrence of these mutants in human colon and breast cancers, can resolve your concern that there was no "causal evidence for tumorigenesis from in-vivo assays." Although our model is consistent (and, indeed, built upon) our understanding of phosphotyrosine displacement, the fact that PTPalpha heterodimerization decreases Grb2 binding was not expected a priori and is an important new finding that should inspire research by a number of groups. (The novelty of this finding is reflected in the fact that no one has previously thought to test the effect of PTPalpha homodimerization on Grb2 binding, even though this phenomenon has been studied for over a decade.) I agree that we do not yet have a "structure-based understanding" of the mechanism accounting for this effect, but this may be a long time coming. (For reference, I note that there is still no structure-based understanding of the effects of PTPalpha homodimerization, even though a half-dozen papers have been published, inching towards this goal, since the discovery of the effect in 1999.) I think that it is important to reveal the finding, and thus pose the question of mechanism, as soon as possible.

Might you reconsider sending our manuscript out for review?

Additional Correspondence 15 November 2010

Thank you very much for your correspondence that essentially confirmed our grasping of the major findings of your study. I also understand that you may have by now essential and in-vivo evidence for these PTPalpha245-truncations to act highly tumorigenic. Appreciating the potential general occurrence of this kind of mutation as shown in actual patient-derived tumor tissue, I would be willing to have it reviewed by experts in the field.
Please not however, that the mechanistic concerns remain rather valid and though appreciating that initial functional observations might spark recurrent interest to elucidate mechanistic detail within the framework established more than ten years ago, it is hard to predict what impact this limitation might have on the peer-review process particularly for a journal that has a strong reputation for molecular- mechanistic insight.
Based on these notes I will leave the final decision whether to resubmit to The EMBO Journal or rather go elsewhere entirely up to you and promise to arrange for peer- review should you be convinced the paper can be strengthen to not waste your's or valuable reviewers time.
I am sorry to be unable to reach a more encouraging decision. I still hope that this letter has clarified our demands and look forward to hear from you soon.

Yours sincerely,

Editor
The EMBO Journal

Resubmission 01 December 2010

Pursuant to our correspondence, we are submitting a revised manuscript.

Src is activated in large fractions of colon and breast tumors without mutation, suggesting that aberrant action of an upstream regulator is involved. We have previously shown that RNAi of PTPα reverses Src activation and induces apoptosis in human colon and estrogen receptor-negative breast cancer lines (but not in normal breast cell lines) suggesting that PTP is the activator in these tumor types. Here this hypothesis was supported, with PTPα activation explained by mutation, by the detection of PTP mutants in 30% of human tumors of these (i.e., colon and breast) types and only in 1 of 19 tumors of other types and in 0 of 36 patient-paired normal tissue samples. One mutant, PTP 245, which was found in colon, breast and liver tumors, was studied extensively and shown to be an oncogene: its expression in human tumor cells correlated with tyrosine-dephosphorylation (and thus, presumably, activation) of Src, it transformed rat embryo fibroblasts to focus-formation and anchorage-independent growth, and induced tumors in vivo in a xenograft nude mouse assay.
Moreover, the molecular mechanism by which PTPα245 induces transformation is novel. Surprisingly, it (like the other splice mutants) lacks the PTP catalytic domain and activates Src by an unusual mechanism: It heterodimerizes with endogenous PTPα(ePTPα), which is co-expressed in the transformed cells, thereby reducing wt PTPα's ability to bind Grb2. Since Grb2 binding inhibits the ability of PTPα to perform phosphotyrosine displacement, which is specifically required for dephosphorylation of Src family kinases, heterodimerization increases ePTPα dephosphorylation of Src but not of other substrates.
Admittedly, the structural basis for the heterodimerization-induced reduction in Grb2 binding is not known. However, this reduction may be related to the previously reported reduction in PTPα-Grb2 binding during mitosis (Zheng et al., 2001) and the increase in wt PTPα 's ability to dephosphorylate Src upon release from homodimerization (Jiang et. al, 1999). While, even after a decade, the structural basis for these effects is also not known, these new results suggest new hypotheses that may finally allow this puzzle to be solved.

The escape of the premature termination codon-containing splice mutants from nonsense mediated mRNA decay (NMD) was unexpected, but not without precedent. Interestingly, this finding is complemented by bioinformatic analyses that suggest that the mutants may be splice variants that play normal roles in other tissues or developmental times. Thus, this may be a case of tissue-specific escape, a possibility that will impact the alternative splicing and NMD research community.

In addition, we expect that the clinical implications of these results will motivate investigations of the potential for PTPα in molecular diagnosis and therapy. While we intend to pursue related studies within the context of basic research, we believe that it is important to bring these findings to the attention of the clinical research community, which can expand research in this area, as soon as possible.
There are no conflicts of interest.

Note that while this study uses human tumor material, the material was obtained from existing clinical samples from operations performed solely for the benefit of the patient. Also, no information regarding the identity of the patients was provided to us. Thus, both Shanghai JiaoTong University and Cornell have determined that this is not human subjects research. Substantiating documents can be provided.

2nd Editorial Decision 03 January 2011

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office.

As you will see from the comments enclosed, the referees appreciate the interesting novel proposal for that role of PTPRA splice variants in tumor progression. However, referee #2 raises a very critical point despite the discovery of such splice variants in tumor tissue the actual contribution to human tumorigenesis is never experimentally demonstrated. This current disconnect leads referee #2

to question suitability of the dataset for publication in a more general journal as The EMBO Journal. Given the otherwise mostly positive comments, we would like to offer you the chance to expand the study according to the referee comments during a single round of major revisions. Please note that validation in human tumor cell lines as requested by ref#2 would be essential for further consideration here. I thus urge you to take his/her comments serious and invest the necessary time and experimental efforts to convince this referee from the significance of your findings but also to avoid later disappointments. To facilitate this, we are able to grant additional time for necessary experimentation upon authors request.
Finally, I do have to formerly remind you that it is EMBO_J policy to allow a single round of major revisions and that the final decision on acceptance or rejection entirely depends on the content and strength of the final version of your manuscript. In case of further questions please do not hesitate to contact me directly preferably via E-mail.

Looking forward to assess a future revised version.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

Huang et al. identified alternatively spliced forms of PTPalpha in tumors at relatively high incidence (6/36). Three different splicing changes were observed, all leading to deletion of D1, that contains the catalytic activity. The most common alternatively spliced isoform occured in three tumors and results in insertion of a cryptic exon and hence to truncation of PTPalpha protein to 245 residues. Truncated PTPalpha245 is detectable in tumors and Src phosphorylation is reduced in these tumors, consistent with activation of Src. Expression of PTPalpha245 in REF cells results in anchorage-independent growth, tumor formation in nude mice and activation of Src, which is dependent on endogenous full length PTPalpha (ePTPalpha). PTPalpha245 heterodimerizes with ePTPalpha, which in itself does not affect catalytic activity of ePTPalpha. A PPLP motif in the juxtamembrane domain of PTPalpha is required for the interaction. PTPalpha245 expression results in reduced GRB2 binding to endogenous PTPalpha. Whereas the mechanism of alternative splicing remains to be determined, the cryptic exon that results in PTPalpha245 is highly conserved in mammals. The authors conclude that PTPalpha245 occurs frequently in human tumors and is able to activate Src, suggesting that it participates in carcinogenesis.

This is an interesting paper describing a novel mechanism for Src activation in tumors. The mechanism underlying alternative splicing is not addressed, but the mechanism of Src activation is explored thoroughly. The data presented here provide an explanation for why Src activity is elevated in many tumors even though no mutations were found in Src in these tumors.

Points:
1. This paper builds on a model that was proposed previously by this research group in which the C-terminal pTyr789 in PTPalpha binds the Src SH2 domain and displaces Src pTyr530 (p.3). Several reports have demonstrated that mutant PTPalpha-Y789F still activates and binds Src and that the Src SH2 domain is not required for binding to PTPalpha (e.g. Lammers et al. 2000 JBC 275: 3391; Yang et al. 2002 JBC 277: 17406; Chen et al. 2006 JBC 281: 11972; Vacaru et al. 2010 MCB 30, 2850). This should be discussed.

2. p.11. The authors argue that the frequency of the occurence of the splice mutants is relatively high (14-69% of colon tumors, 95% Wilson confidence interval). To back up this conclusion, they claim that the frequency is only significantly exceeded by the very high mutation rate of APC in colon cancer. This is an overinterpretation of the limited number of tumors that were examined and I suggest to delete the remark about the mutation rate of APC.

3. p. 15. The mechanism underlying alternative splicing of PTPalpha in tumors remains to be determined. Whereas elucidation of this mechanism is perhaps beyond the scope of this paper, the authors indicate in the discussion that PTPalpha245 apparently escapes nonsense mediated decay. The authors should extend the discussion here and address the following important questions: Is nonsense mediated decay regulated in cancer? Are the alternatively spliced PTPalpha isoforms cause or consequence of the tumors?

4. Fig. 5. (p.8) Roughly equal amounts of PTPalpha and PTPalpha245HA were detected in immunoprecipitates. This suggests that PTPalpha245 and ePTPalpha heterodimerize roughly in a 1:1 ratio. Several reports have demonstrated that the ectodomain and transmembrane regions of PTPalpha contribute to dimerization (Jiang et al., 2000; Tertoolen et al., 2001). Co-expression of full length PTPalpha with a deletion mutant (lacking most of the cytoplasmic region of PTPalpha) results in homo- and heterodimerization (Tertoolen et al., 2001). Therefore, the ratio of heterodimerization of PTPalpha245 and ePTPalpha is maximally 3:2 instead of 1:1. In addition, the heterodimers have to be extremely tight (very high affinity) to reach the maximal ratio of 3:2. It is more likely that even less ePTPalpha is co-immunoprecipitated with PTPalpha245. This should be explained. Along these lines, the results of the PTP assays in Fig. 5B are puzzling: the origin of the PTPalphaHA bars are not described at all. Presumably, this is a control transfection of full length PTPalphaHA? If so, given the heterodimerization of full length PTPalphaHA with ePTPalpha, the high salt wash should lead to a reduction in PTP activity, resulting from the release of ePTPalpha. This was not observed here and should be explained.

5. p.12, 13. The authors propose two possible models: (1) GRB2 does not bind to full length PTPalpha dimers, resulting in increased binding and activation of Src, (2) GRB2 binds to both full length PTPalpha molecules in the homodimer, explaining reduced GRB2 binding to PTPalpha245 (Fig. S2). The latter model is explained in more detail in the supplementary material and I suggest to include Fig. S2 in the manuscript proper, because this model is most appealing in my view.

Referee #2:

The manuscript by Huang et al. presents original data suggesting that at least one splice isoform of PTPRA (PTPalpha) encodes a truncated version of this PTP that acts by dimerizing with, and activating the WT endogenous PTPRA. Hence, this activation of the PTP causes the dephosphorylation of the human tyrosine kinase c-Src on the 530 p-Tyr (the negative regulatory p-Tyr). This event leads to the activation of c-Src enzymatic activity and its potential oncogenic effects. The authors suggest that targeting PTPalpha and the 245 splice form would be an excellent approach in specific cancer therapies.

Overall the mechanism proposed is original and brings a new interesting way by which expression of PTPRA and/or its splice isoforms can cause cancer. However, several questions are raised by their findings, which will require further work to support their assessment.
Among others,
- A main concern is that the justification for the proto-oncogenic cascade induced by the 245 splice form towards cancer is weak and that the model presented is itself not well supported.
- PTPRA has been associated with oncogenic activity in head and neck cancers but was inversely correlated with aggressive breast cancers, supporting that this receptor was acting as a tumor suppressor in breast cancer and not as an oncogene.
(Ardini, E. et al. Expression of protein tyrosine phosphatase alpha (RPTPalpha) in human breast cancer correlates with low tumor grade, and inhibits tumor cell growth in vitro and in vivo. Oncogene 19, 4979-4987 (2000).
- The crystal structure model used for their model is itself debated and there are no strong data supporting in this manuscript this model instead of the head-to-toe presented by Barr et al. (2009). The authors propose a tetrameric structure complex with two c-Src and a homo or heterodimers of PTPalpha wt or spliced forms... Much is debated in the literature on the dimerization of the D1-D1 complex versus the Head-and -toe model. This should be at least discussed by the authors. Fig. S2 as depicted does not reflect the mechanism of Grb2 displacement that they describe in the discussion.
- The title is inappropriate as the authors never demonstrated that the 245 isoform contribute to

human tumorigenesis.
- The data from human tumors depicts a relatively rare number of tumors (breast and others) that have the splice isoforms described (RPTP alpha 245).
- Importantly, no human cancer cell lines expressing the isoforms were employed to knockdown RPTPalpha wt or the 245-splice variant to validate the contribution of this isoform to the oncogenic properties of the selected human cancer cells.
- Similarly, as the model includes a possible and important contribution by Grb2, what would happen in Grb2 knockdown by SiRNA/shRNA?
Specific Comments

Abstract:
The authors are quite imprecise in their statement "Yet Src tyrosine
phosphorylation was greatly reduced (implying activation)" this phrase must be associated with the particular C-terminal pTyr site which is a negative regulator of Src activity. Similar statements are made throughout the manuscript; the phosphorylation status should be associated to a specific site and tested with the proper phosphospecific c-Src antibodies.

In addition the manuscript should use the nomenclature of PTPRA (RPTPalpha). At the minimum the two names should be included in the abstract.

The RPTP alpha 245 must be knock down by siRNA/shRNA in human tumor cells expressing the splice isoforms in order reverting the transforming phenotype in these cells and thus to validate the contribution of the isoforms to cancer.

Since the RPTPalpha 245 has a unique sequence a specific antibody could be made towards this sequence in order to confirm that the lower form is indeed this unique splice form. Does the HA tagged 245 isoform localizes to the cell membrane? Does it co-localize with wt PTPRA?

The use of the c-Src pY-530 antibody and the kinase activity with enolase as substrate should also have been supported with antibody against the p-Tyr 418 sites.

The model of interaction between c-Src and the homodimer/heterodimer remains also to be examined further. For example the authors uses the structure proposed by Bilwes et al., 1996 describing a helix-turn-helix wedge of one D1 inserting into the active site cleft of the other D1. Yet, this structure is quite controversial as last year paper by Barr et al. Cell 136: 352-363, 2009 state that this is more likely a head to toe organization and that in liquid PTPRA D1 domains in fact did not dimerize.

Secondly, the binding of the Src SH3 domain on the D2 of PTPRA is also not determine clearly. Is there a specific Pro rich domain recognized by the SH3 of c-Src? Finally, the model proposed presents a heteromeric complex of two c-Src and two PTPRAs. Is this the case? In non-denaturing gels does this tetrameric complex exist? With WT PTPRA? What is the complex formed with the alternative splice form? How does c-Src activated if it does not bind the PTPRA wt-245 heterodimers.

It is not clear in the Mat. and Methods if the re-expression of the PTPRA WT and various isoforms are human cDNAs. Moreover, most of the experiments of reconstitutions are performed in rat fibroblast. Is it known that human PTPRA dimerizes with the endogenous rat PTPRA?

Use of brackets and parenthesis should be discouraged in the manuscript; [a role suggested for FAK in integrin stimulated fibroblast focal adhesion plaque complexes (Chen et al, 2006)]; (Insufficient tumor tissue remained to directly measure Src specific activity.); and many more...

The authors should state clearly in the results section that all mRNA from the tumors were reverse transcribed using the oligo dT primers. The existence of these alternative splice forms is crucial for the validity of the manuscript. Do the altered splicing PTPRA forms are poly adenylated? Can these mRNAs be found associated with loaded ribosomes? What about the protein expression?

Fig 2. The author should include a modified Fig2 with a new panel A that depicts all of the intron-exon of PTPRA and the three different alternative splice form uncovered by the authors. Then

modify the current panel 2A and 2B to 2B and 2C to present the detailed of the cryptic donor/acceptor sites and the resulting amino acid sequences of the alpha245. This figure is important and should be done in a way to be more informative to the readership.

Fig 3. Did the specific c-Src p-Tyr 418 and/or 530 antibodies used on the human tumor samples? Which pTyr antibody was used? Again the authors do not clearly describe which antibody and site that they are using. The sample of colon, breast and liver come from which patient numbers? On which basis the authors state various percentage of decrease or increase in Src phosphorylation. Did the authors scan their western blots? This should be included in the data. Otherwise all western blots should be scanned to estimate the levels of Src phosphorylation.

Fig.4. What is the effect of wt exogenous PTPRA?

Fig.5 The full length PTPRA PPLL mutant should also have been included in the assay.

Fig. 6 Does the anti- pTyr only recognizes PTPRA Tyr-789?
The Grb-2 IB from Grb-2 IP is over exposed. However, it seems that there are more Grb-2 in the total extract in the dox treated cell extracts.

Referee #3:

This is an important study that provides new mechanistic insights into the functional interplay between a receptor protein tyrosine phosphatase, PTP-alpha, and the oncoprotein tyrosine kinase SRC. The authors present several significant novel findings. They identify novel splice variants of PTP-alpha from colon, breast and lung tumors; these are truncations that lack the catalytic domain and they focus on one, PTP-alpha245, to define its mechanism of action. They demonstrate that this catalytically inactive form of PTP-alpha transforms rat embryo fibroblasts and generates tumors in xenograft studies in nude mice. The authors reveal an interesting mechanism of action of PTP-alpha245 that integrates several important areas of research in the signaling function of PTPs. They show that PTP-alpha245 heterodimerizes with endogenous full-length PTP-alpha (ePTP-alpha), which results in a decrease in the association of GRB2 with ePTP-alpha, which in turn promotes the dephosphorylation and activation of SRC. This provides further support for the importance of receptor PTP dimerization and for the pTyr-displacement mechanism for activation of SRC by PTP-alpha. Overall, this is a terrific manuscript, not only for those interested in PTPs and regulation of tyrosine phosphorylation, but also those interested generally in signaling and cancer. I recommend that it should be accepted after attention to the following minor points.

I recommend that the authors should deal further with the issue of regulation of SRC by tyrosine phosphorylation, as I found this aspect confusing. The model, as I understand it, is that there is an inhibitory site of phosphorylation at the C-terminus of SRC, which is the site of action of PTP-alpha; however, there is also an autophosphorylation site (Tyr 416) in the activation loop that is associated with the active form of the enzyme. The authors report that phosphorylation of SRC decreases as a result of PTP-alpha action (Fig 3) - what is happening to Tyr 416 in these experiments?

I think it would be helpful to show the state of tyrosine phosphorylation of PTP-alpha in the experiment in Fig 6 that examines GRB2 association - i.e. look at both Tyr phosphorylation and GRB2 association together in the same experiment.

The Discussion is a little long and meandering and would benefit from tightening up. It is not clear to me that Fig 7 and all the discussion of that figure really helps the main message of the manuscript regarding PTP-alpha, SRC activation and cancer. Given recent publications on the importance of splicing and splice factors in tumorigenesis, is there anything that the authors can say about the splice factors that generate these forms of PTP-alpha? I think the importance of PTP-alpha in breast cancer is not quite as well established as the authors suggest.

Yours sincerely,

Editor
The EMBO Journal

1st Revision - Authors' Response 01 April 2011

It appears that the referees appreciate the significance of our results in the context of signal transduction, but feel that the relevance to human cancer has not been adequately demonstrated. Referee 3 states "This is an important study that provides new mechanistic insights...[and which] present[s] several significant novel findings" and "Overall, this is a terrific manuscript, not only for those interested in PTPs and regulation of tyrosine phosphorylation, but also those interested generally in signaling and cancer," referee #1 states "This is an interesting paper describing a novel mechanism for Src activation ...[which] is explored thoroughly," and referee #2 states "...the mechanism proposed is original and brings a new interesting way by which expression of PTPRA... splice isoforms can cause cancer." We agree with the referees' primary reservation, that the relevance to human cancer has not been sufficiently demonstrated, but also believe that the biochemical, tissue culture, and nude mouse model results, which demonstrate the unexpected ability of a phosphatase-defective RPTPα truncation mutant to activate Src by a previously unsuspected mechanism, are important on their own and merit publication at this time. A study of the causal role of the mutants in human carcinogenesis must await establishment of human tumor lines that express them, which will require at least a year (see below). It would be unfortunate to withhold our current results for this length of time.
To emphasize the biochemical/tissue culture focus of the paper, we have modified the title, abstract and discussion. Moreover, throughout the text we have greatly "toned down" the suggestion that there may be a causal role in human tumors and leave this as a speculative possibility.

I apologize for the delay in returning our revised manuscript. In response to Referee #3 (point #2) I had hoped to include an anti-pTyr immunoblot in a repeat of the experiment of Fig. 6 (to supplement the anti-pTyr immunoblot shown in Fig. 5) before the three-month response period was over. However, because I have had to temporarily return to China and due to delays in receiving some needed commercial reagents, this has not been completed and I am submitting our revision with the request that you reconsider it for acceptance at this time. As discussed below, this internal control would provide a more elegant presentation, but I do not think that it is essential. If you disagree and the manuscript is otherwise acceptable, we should be able to replace Fig. 6 soon.
Regarding studies with human tumor lines expressing RPTPα245 or other RPTPα splice mutants: We have already used cDNA sequencing to screen the RPTPα mRNAs in 3 colon cancer lines and 8 ER and 4 ER+ breast cancer lines without finding any splice mutants. It is possible that this is simply bad luck (as mentioned in the text, the lower confidence limit on splice mutant frequency is ~13%), but alternatively could indicate that the splice mutants occur predominantly in specific genetic backgrounds. All tumors were from patients of Chinese descent but only one of the 15 cell lines was from an "East Asian." We are currently getting the only other available (to our knowledge) Asian breast cancer line for testing. [As of yet, we have been unable to aquire three newly-established Chinese breast cancer lines (Shen et al., 2009, Cancer Cell Int., 9:2).] Thus, it appears that we will have to make additional Chinese lines to get sufficient statistics. It will also be important to screen human tumors from non- Chinese patients. In addition, we do not rule out the possibility that RPTPα splice mutant expression might be lost during the establishment process because of changes in transcription rate or other factors that modulate alternative splicing (Nilsen & Graveley, 2010). Even if RPTPα245-mediated activation of eRPTPα contributes to tumorigenesis, lower eRPTPα activity might be sufficient in the cell line because of other well-known changes that usually accompany establishment. Thus, without selection pressure, the splice mutant might be lost, particularly since most of these lines have been in culture for decades and many were derived from tumor metastases, not from primary tumors (Burdall et al., 2003, Breast Cancer Res, 5:89). Therefore, if we do not find RPTPα splice mutants in existing cell lines, we will address this question in the future by simultaneously using the same (unfrozen) Chinese tumor sample for cell line establishment and for cDNA sequencing. This will either yield cell lines expressing the splice mutant or demonstrate the loss of the splice mutant during the process of establishment, setting the stage for studies to understand the transcriptional processing changes involved.

We describe the need for additional screens with non-Chinese patients in the paper, but do not present the full discussion above as it is a digression from the main focus.

Specific responses to the referees' comments:

Referee #1

1) Activity of Y789F mutants:

Some care is required in interpreting experiments testing the effect of Y789F mutation on the ability of PTPα to activate Src and transform cells. Our original manuscript cites the suggestion of Chen et al (2006) that third-party proteins can act in lieu of pTyr789 to provide phosphotyrosine displacement following integrin stimulation and that this might also be true in the neuronal system of Yang et al (2002). The papers by Kapp et al* (2007) and Vacaru and den Hertog (2010) are consistent with the interpretation that Y789F mutation reduces but does not eliminate RPTP 's activity on Src. They do not conflict with the model, which only requires that phosphotyrosine displacement regulates RPTPα activity, not that it be the only regulator. We now include references to all of these papers, emphasize that factors beyond the decrease in Grb2 binding might also be involved, point out that changes in Ser phosphorylation and/or D2-Src binding could also be involved, and emphasize that tests with cell lines established from human tumors expressing RPTP 245 will be needed to resolve the question. We have also added a Supplementary Discussion that better explicates this complex issue.
*The referee refers to Lammers et al (2000), but that paper does not demonstrate activation of Src by RPTP (y789F). Kapp et al (2007), a subsequent paper from Lammer's group, does and so we reference this paper in this context. Lammers et al (2000) is discussed in the Supplementary Discussion.

2) *Delete remark about mutation rate of APC*:

The suggested change has been made.

3) *Causal role of altered splicing and NMD?*

Text and additional references have been added to the Discussion and to a new Supplementary Discussion to address these points. We emphasize that determining whether the mutations are epiphenomena or causes of the tumors is a critical question and that cell lines will have to be established to answer it. The possibility that changes in the tumor microenvironment might affect NMD but that there is, as of yet, not evidence linking inhibition of NMD to tumor formation is discussed in the Supplementary Discussion with a review reference.

4) *Quantitation of RPTP 245-eRPTPα heterodimerization in Fig. 5A:*

The goal of these experiments was to demonstrate the significant non-covalent association between RPTP 245 and eRPTPα that explained the RPTP 245-associated PTP activity, not to precisely quantitate the effect since even wild-type RPTPα homodimerization has not been quantitated by previous study. The only quantitation available is by densitometry of scanned autoradiographs and the measurements are subject to film nonlinearities and may not be quantitatively accurate. Thus, we do not think it is appropriate to present a quantitative analysis in the manuscript. Here we present a semi-quantitative analysis that shows that the observed results are reasonable and consistent with the observations of Jiang et al (2000) and Tertoolen et al (2001). These papers show that the transmembrane domain is sufficient for some RPTPα association and that the extracellular, wedge-D1 and D2-D2 interactions also contribute. However, neither paper presents the information needed for quantitative modeling (i.e., the fraction of eRPTPα that is normally homodimerized, quantitative measures of the relative contributions of the different domains to binding, and whether the different domains contribute independently to binding or whether, as seems most likely, there are cooperative nonlinear contributions to the free-energy of binding). Thus, this analysis uses reasonable, but currently unsubstantiated, assumptions. It concludes that the observed results would be expected if RPTP 245-RPTP 245 homodimerization is very weak and if RPTP 245-eRPTPα heterodimerization

is half as strong (twice the Kd) of the eRPTP -eRPTPα homodimerization. We now summarize these points in the text as requested by the referee. However, since the full quantitative analysis is based on presumptive values, which have not been experimentally measured, we do not think it appropriate to include it in the manuscript.

Semi-quantitative modeling of RPTPα245-eRPTPα heterodimerization:

We assume that dimerization is described by simple equilibrium binding parameterized by dissociation constants Kww (eRPTPα-eRPTPα homodimerization), Kwm (eRPTPα-PTPα245 heterodimerization), and Kmm (RPTPα245-RTPTα245 homodimerization). This greatly oversimplifies this complex membrane-associated process, which may involve cooperative effects with third-party proteins, but provides a useful feasibility demonstration.
Densitometry of the HA IP/RPTPα IB co-IP experiments (Fig. 5A lanes 1-4), with correction for the difference in antibody reactivity to eRPTPα and RPTPα245HA (described in the Fig. 3 legend) shows that 70-100% of the RPTPα245HA is heterodimerized with eRPTPα. We use 70% for this analysis. The converse experiment indicates that ~35% of the eRPTPα is heterodimerized with RPTPα245HA (lanes 5-8), corresponding to a RPTPα245HA expression level of ~0.5 (~0.35/0.7), using units where the eRPTPα expression level is 1. Consistent with qualitative published data, we assume that almost all (95%) eRPTPα is homodimerized in the absence of RPTPα245. This implies that Kww~0.005 [=0.05^2/(0.95/ 2)]. We assume that eRPTPα-RPTPα245 binding is 3x weaker (Kwm~0.015) (because the D2-D2 interaction is missing and only one D1-wedge interaction is present) and that RPTPα245-RPTPα245 homodimerization is very weak with Kmm~3. Solving the standard equilibrium binding and mass conservation equations gives [eRPTPα-eRPTPα]~0.3, [eRPTPα-RPTPα245] RPTPα245]~0.35, and [RPTPα245-RPTPα245]~0.04. This predicts that eRPTPα/RPTPα245~0.7 in the anti-HA IP/anti-RPTPα IB experiments and that RPTPα245/αRPTPα~0.35 in the anti-D2 IP/anti-RPTPα IB experiments, which matches the observed results.
"... the origin of the PTPαHA bars are not described...": As surmised by the referee, these were control lanes using cells that stably overexpressed RPTPαHA. This omission has been corrected in the figure legend and in the text.
"...given the heterodimerization of full length PTPαHA with ePTPα, the high salt wash should lead to a reduction in PTP activity, resulting from the release of ePTPα. This was not observed here and should be explained.": We believe that this is explained by the inability of the single 1 min 0.5 M NaCl wash that was used to dissociate the wild-type dimers. Since any antibodies that recognize eRPTPα also recognize RPTPαHA, we are unable to directly measure the amount of dissociation. However, quantitation of the eRPTPα-RPTPα245HA immunoblots (Fig. 5A) shows that about 15% of the eRPTPα-RPTPα245HA heterodimers survived the high-salt wash. This is consistent with the amount of PTP activity retained (Fig. 5B). The additional wedge-D1 and D2-D2 binding in the eRPTPα-RPTPαHA heterodimers are expected to further stabilize binding, so they may have been resistant to the wash. This is now discussed in the text.

5) *Include Fig. S2 in the manuscript proper:*

We agree with the referee that this model is the most attractive explanation of the results and, accordingly, have reordered the discussion so that it comes first. However, it is still speculative and so it gives it overmuch weight to include Fig. S2 in the text. In particular, Referee #2 appears to hold a contrary opinion, does not appreciate this model as much, and might object to including it in the text. We defer this choice to the editor. If he thinks it appropriate to include it, it would be a simple matter to move it and the associated discussion into the main manuscript.

Referee #2

1) *Justification for the proto-oncogenic cascade induced by the 245 splice form towards cancer is weak:*

As discussed at the beginning of this response we agree and have modified the title, abstract, and discussion to de-emphasize this point.

2) *Inverse correlation of RPTPα expression with aggressive breast cancer*:

We have previously shown that RPTPα plays different roles in estrogen receptor negative (ER) and ER+ breast cancer lines (Zheng et al, 2008): it is required for Src activation and cell survival in ER lines, but not in MCF7 and other tested ER+ lines. This is not inconsistent with by Ardini et al's demonstration that overexpression of RPTPα inhibits growth of MCF7 cells. Also, since the ER+: ER breast cancer ratio is ~ 4:1 in breast cancer in Caucasion and Asian populations (Hausauer et al, 2007), the correlation that they noted between RPTPα expression and low tumor grade in all breast cancers probably describes a property of the dominating ER+ subtypes. This point was previously discussed in Zheng et al (2008) and is now discussed here as well. 3a) Barr et al model for structure: We are not convinced by the arguments presented by Barr et al that the wedge-D1 interaction does not occur in RPTP .

Specifically: (1) Their primary argument was that this is prevented by a steric clash inferred from superposition of the RPTPα D1-D2 dimer structure and the assumption that the D1-D2 linkage in RPTPα is rigid (their Fig. S2). However, the RPTPα rotational changes observed following oxidative stress suggest that the linkage is not rigid (Blanchetot et al, 2002; van der Wijk et al, 2003).

(2) The D1-wedge interaction of the Bilwes structure explains the observation by Jiang et al (1999, 2000) that Pro210 Leu/Pro211 Leu mutation greatly weakens RPTPα dimerization. These residues face outwards in Barr's head-to-toe structure and it is hard to envision why, in this conformation, the PPLL mutation would have any effect.

(3) Jiang et al (1999) show that mutagenic introduction of a disulfide bridge between the extracellular domains of two RPTPα molecules suppresses RPTPα activity only when the bridge is introduced at a position (Pro135) consistent with the wedge-D1 interaction observed by Bilwes et al. It is not evident why there would be such positional sensitivity (e.g., disulfide bridges at 131 and 141 do not suppress activity) nor why disulfide bridges would form at all in the head-to-toe conformation. (3) Amino acid conservation at the RPTPα head-to-toe dimer interface is 100% to RPTPα, suggesting (as posited by Barr et al) that it forms dimers in the same manner. However, the conservation to RPTPα is only 45% (=5/11) and 4 of the changes are (non-complementary) changes in charge. (4) The head-to-toe dimer puts the two transmembrane domains noncontacting and far apart (see Fig. 7, Barr et al). This seems inconsistent with the required role of the transmembrane domain in RPTPα dimerization (Tertoolen et al, 2001, Fig. 5). The first two points are now included in the discussion. We also point out that the cross-binding model can operate with either head-to-head or head-to-toe homodimerization. The only change would be a redrawing of Fig. S2.This is now noted in the Fig. S2 legend.
3b) Fig. S2 does not reflect the mechanism of Grb2 displacement: The comparison of panels a and b is designed to show that Grb2 can only bind the monodimer because RPTPα245 lacks the D1 region needed for Grb2 C-SH3 docking in the cross-binding. This point is now emphasized.

4) *Inappropriate title:* We have changed the title to make it clear that we are only reporting the source of the mutant in mentioning human tumors.

5*) Relatively rare number of tumors*: The observed occurrence rate (30%) of RPTPα truncation mutants in the a priori defined subset of colon and breast cancers (Zheng et al, 2008) is not rare but is large in comparison with other oncogenes that have been implicated in carcinogenesis. As mentioned in the text, statistical analysis implies a lower confidence limit of 13% (= 0.05); even this is still of significant interest. We now strongly emphasize that we do not know whether RPTPα245 plays a causal role or is only a secondary effect of carcinogenesis and that this can be studied once human cell lines expressing RPTPα245 have been established.

6) *No experiments with human cancer cell lines expressing the isoforms:* We completely agree that these experiments are key for testing the carcinogenic role of the truncation mutants in the human cancers. Such cell lines were not made since only frozen human tumor samples were provided for this initial study. We are planning a new study that will use fresh tumor samples; this is logistically more challenging and it will be roughly a year before such lines are available. At that time it will be possible to address the question of causality in humans using knockdown experiments. At this time

our focus is on the mechanism of activation itself.

7) *Effect of Grb2 knockdown: A Grb2 knockdown experiment would be noninformative for the mechanism by which RPTP 245 transforms cells*:

We have shown that Grb2 binding to RPTPα negatively regulates its dephosphorylation of Src (Zheng et al, 2000; Zheng and Shalloway, 2001) and that its binding to endogenous RPTPα is already suppressed in the RPTP -RPTP 245 hybrids (Fig. 6). Therefore, it would not be surprising if knocking-down Grb2 had no effect. Conversely, since Grb2 plays many roles in transformation (e.g., in regulating Ras-mediated pathways that act downstream from Src) it would not be surprising if Grb2 knockdown suppressed transformation. Neither result would tell us anything of interest. Possibly the referee is referring to seeing if Grb2 knockdown changes the amount of RPTPα245-RPTPα heterodimerization. Again, such a measurement would be uninformative: Since most RPTPα245 is heterodimerized with endogenous RPTPα in normal cells (Fig. 5, discussion in text, and response to Referee 1, #4), a further increase would not be detected under any circumstances. Conversely, since the role of Grb2 in cells is so complex, a decrease would also not have a clear interpretation. For example, Grb2 is involved in localizing proteins (as in the Ras-SOS interaction), and could affect RPTPα localization and dimerization in a complicated manner. Observing any change would certainly open more questions for investigation, but would not contribute to this manuscript. For this reason our current and past studies have studied the in vitro interactions of RPTPα and Grb2, which have clear interpretation. We do not believe that an in vivo knockdown experiment with RPTP 245 is worth conducting at this time.

8) *Abstract*: *The authors are imprecise in their statement about Src activation*:
The statement that the reduction in total Src tyrosine phosphorylation in the human tumors implies that Src is activated in these cells is correct. However, as there is no space in the abstract to explain this, it has been removed from there and is explained in the text. The first paragraph of Introduction now explains that RPTPα is most commonly observed to simultaneously dephosphorylate both Tyr419 and Tyr530 of Src which results in ~5x activation (den Hertog et al, 1993; Zheng et al, 2000). Assuming that the decrease in total Src tyrosine phosphorylation was due to such simultaneous dephosphorylation is the most conservative interpretation of the measured decrease of total Src pTyr in the human tumor cells. The decrease in total pTyr would imply an even greater activation if pTyr530 were dephosphorylated, and the resultant autophosphorylation at Tyr519 were not removed, since the amount of pTyr530 dephosphorylation would have had to have been even greater to account for the net decrease. There is no ambiguity to this statement in regard to the activation of Src by RPTP 245 in REFs; this was directly measured using enolase as substrate (Fig. 3B).

9) *Nomenclature:*

PTP , RPTP , and PTPRA have been used by different groups to identify this protein for some time and I am not aware that any consensus has been reached on nomenclature. A PubMed search for the most recent paper having "PTP alpha," "RPTP ," or "PTPRA" in the title yields a 2010 paper titled "PTPα activates Lyn and Fyn and suppresses Hck to negatively regulate Fc RI-dependent mast cell activation and allergic responses" (Samayawardhena and Pallen, J Immunol 185:5993).
None-the-less, we are happy to accede to the referee's request and have changed to "RPTPα," which does seem to be used by the majority of groups.

10) *Knockdown experiments in human cancer cells:* See response to 6) above.

11) *Experiments with an antibody specific for RPTP 245*:

We agree that experiments with a RPTPα245-specific antibody would be interesting and plan such for the future, assuming that a good antibody can be generated. However, the current data is sufficient to leave little doubt that the lower band in the anti-RPTPα immunoblots of human tumor cells (i.e., Fig. 3A) is generated by the RPTPα245 mRNA present in the human cells: (1) The band reacts with anti-RPTPα antibody in clean immunoblots, (2) it is present only in the tumor cells that express the RPTPα245 mRNA and not in the paired normal cells, (3) it has the predicted

electrophoretic mobility, (4) its electrophoretic mobility (and spread due to variable glycosylation) matches that of RPTPα245 that is inducibly expressed under tet-off control in tissue culture (Fig. 3B). The fact that most RPTPα245 co-immunoprecipitates with eRPTPα (see point 4, Referee 1, above) clearly implies that the two proteins must be colocalized.

12) *Use of anti-pY530 antibody should have been supplemented with anti-pY418 antibody:*

As discussed in point 8 above, we do not expect to see any phosphorylation at this site following activation of Src by RPTPα and, even if we did, it would not affect the conclusion that Src is activated due to pTyr530 dephosphorylation following RPTP 245 expression. While more information is always desirable, we believe that this is a low-priority experiment.

13a) *The Bilwes structure is controversial:*

See point 3a above. The Barr et al structure is now discussed and we emphasize that the cross-binding model can function with either the head-to-head or head-to-toe structures.
13b) The binding of the Src SH3 domain on the D2 of PTPRA is also not determine[d] clearly: I do not know to what the referee is referring.The Discussion, cross-binding model, and Fig. S2 all refer to the binding of the Grb2 C-SH3 domain to the D1 domain of RPTPα, which has been reported by den Hertog and Hunter (1996) and Su et al (1996). I am not aware of any reports indicating that the Src SH3 domain has been implicated in binding to RPTPα. In any case, this is not relevant to our model.

13c) *The model proposed presents a heteromeric complex of two c-Src and two PTPRAs*:

I am confused about the referee's intent. The model presents a complex between two RPTPα and two Grb2 (not Src) molecules. This is clearly indicated both in the Discussion, Fig. S2, and the figure legend. Our hypothesis is that Grb2-RPTP association blocks the association with Src, so a tetrameric complex is not predicted and is not shown.

14) *Not clear in Material[s] and Methods if PTPRA WT are human cDNAs*:

We have now made it clear that this is the human short-isoform RPTPα. Is it known that human PTPRA dimerizes with the endogenous rat PTPRA?
Although rodent cells have been used by many labs for human RPTPα studies, I am not aware of any studies that have directly tested this. However, since human and rat RPTPα are 95% homologous and the experiments presented here show that (human) RPTPα245 heterodimerizes with rat eRPTPα, it would be extremely surprising if fulllength human RPTPα did not homodimerize with rat eRPTPα.

15) *Use of brackets and parenthesis should be discouraged in the manuscript*:

We believe that their use helps clarify the logical flow of the argument. We have removed some of them and will be happy to accept any additional editorial emendations in this regard.

16) *The authors should state clearly ...that all mRNA ...were reverse transcribed using the oligo dT primers*:

This is now stated in Results and in Materials and Methods. The fact that they were generated using such primers implies that they were polyadenylated. We did not test (presumably by subcellular fractionation) if they are associated with "loaded ribosomes." RPTP 245 expression in the human tumor cells is demonstrated in Fig. 3A and, as reported in the text, was almost equal to that of eRPTPα (>= 80% the level).

17) *Include a new panel in Fig. 2*: This has been done.

18) *Fig. 3 questions*.

The anti-phophosphotyrosine antibody was anti-p-Tyr-100 from Cell Signalling Technology. We have now added the product number (#9411) to Materials and Methods. The patient numbers are the same as in Fig. 1 and have now been duplicated in this figure. The change in Src phosphorylation was determined by scanning of the immunoblots. This is now stated in the text.

19) Fig. 4. What is the effect of wt exogenous PTPRA? Fig. S1 and Table S2 show that the anchorage independent growth induced by wt RPTPα is the same as that induced by RPTPα245. The relevant section of Results has been rewritten to emphasize that the transforming activities of RPTPα245 (induction of focus formation, anchorage independent growth, and in vivo tumorigenicity) are similar to those of wt RPTPα.

20) *Fig. 5. Full length PTPRA PPLL should also have been included in the assay:*

The co-immunoprecipitation assays use the difference between their lengths to distinguish between RPTPα245HA and co-immunoprecipitated eRPTPα. If full length RPTPα(PPLL)HA had been used we would simply have seen a band at the full-length electrophoretic mobility and would not have been able to tell whether this was coimmunoprecipitated eRPTPα or anti-HA immunoprecipitated RPTPα(PPLL)HA.

21) *Fig. 6. Does the anti-pTyr only recognize PTPRA Tyr-789?*

Figure 6 does not involve anti-pTyr antibody. Perhaps the reviewer is referring to Fig. 5. Yes, anti-pTyr antibody only recognizes RPTPα pY789 (Zheng et al, 2000). This is now stated in the figure legend. Scanning showed that the total amounts of immunoprecipitated Grb2 were essentially the same in the Dox+ and Dox- cells.

Referee #3

1) *Clarify role of Tyr 416:*

Please see the response to Referee # 2, point 8, above. The revised Introduction now discusses the role of Tyr416 in Src activation and the fact that it has not been observed to be phosphorylated in RPTPα-transformed cells.

2) *Anti-pTyr in Fig. 6*:

We agree that it would be optimal to have an anti-pTyr blot on eRPTPα included with this experiment. However, as multiple anti-pTyr blots on eRPTPα in other experiments have shown that there is no change in the pTyr789 phosphorylation level (e.g., see Fig. 5A). So we do not expect any surprises. As mentioned at the beginning of this response, I am currently in China (and this experiment must be performed at Cornell) so we will not be able to repeat this experiment including this control for at least a month. To save time, I am returning our response now, but will be happy to include an improved figure as soon as it is available.

3) *Discussion needs tightening up:*

We have tightened up the original Discussion. However, as additional material has now been added in response to referees' requests (see above), the new Discussion is about the same length as the old one.

4) *Fig. 7:*

An important "next step" is to determine the mechanism responsible for generating this alternatively spliced mutant. Fig. 7 is important in this context, provides the most relevant information regarding

the referee's point #5 below, and will help guide future studies. To shorten the Discussion we have moved most of the discussion of this figure to the Supplement. Fig. 7B has been modified to improve clarity.

5) *Splice factors:* Fig.7 and the associated bioinformatic analysis suggests that RPTP 245 may result from inappropriate inclusion of an newly discovered, alternatively spliced exon (cx95) that has a normal role at specific times and/or tissues. Review articles that discuss the attempts and difficulties in determining the significance and mechanisms of alternate splicing in cancer are now cited in the Discussion.

6) *Importance of RPTPα in breast cancer not quite as well established as the authors suggest*:

We agree that the role of RPTP in breast cancer is contextdependent, and in particular depends on ER status (see response to Referee #2, 2). However, the induction of apoptosis by RPTP RNAi in ER cell lines (Zheng et al, 2008) provides strong evidence for a causal role. In any case, as discussed above, the revised manuscript makes it clear that further studies are needed to examine this possibility.

3rd Editorial Decision 15 April 2011

Thank you very much for submitting a revised paper. Upon detailed in-house assessment, I decided to consult with an external advisor, instead going back to the original referees. This decision was based on the relatively polarized initial comments (with two referees being positive and one having strong objections), thus offering a fourth, unbiased judgement. Having received confidential comments, I am unable to transmit them in full, but can reassure you that these were overall rather positive. However, a few minor questions were raised that I would like to offer you the chance to consider/respond to these valid points on before eventual acceptance of an ultimate version of your paper:

1) related to the c-Src Tyr phosphorylation status: the rebuttal arguments are appreciated, and the c-Src kinase assays seem to establish that c-Src is activated in cells expressing RPTPα245. Still, inclusion of anti-pY416 blots to score the active form of c-Src would be much more convincing.

2) The information on siRNAs used should be presented in the main body of the text. If the RPTPα245 mRNA simply contains the additional cryptic exon, then it should contain all the downstream exons present in the mRNA for full length RPTPα. It such seems not entirely clear how selectively targeting of full length RPTPα could be achieved with siRNAs.

3) Colony-forming assays: these seem to have been performed with siRNAs rather than stably expressing shRNAs. Being scored after 21 days in agar, raises the question on the lasting siRNA effects. Would one not anticipate re-expression of the targeted mRNA after a few days? Please comment.

4) No actual data on WT RPTPa stable homodimers are presented. If there are RPTPa homodimers in the IP's and they really have the same activity as the RPTPa/RPTPa245 heterodimers, one would have to conclude that the full length RPTPa homodimers are not in the wedge-mediated inhibitory configuration in the IP. This deserves at least some comments. Functionally, a lot depends on the 32P-MBP phosphatase assay, and it is not exactly clear how linear this is.

5) Related to decreased binding of Grb2 to RPTPa in cells expressing RPTPa245: this seems reasonably well documented, but whether this is the cause of c-Src activation by RPTPa is not established. The argument that depleting Grb2 might not give a meaningful result seems valid, but effects of overexpressing Grb2 to increase binding to heterodimers could be informative/supportive.

I am very much looking forward to your response.

Yours sincerely,

Editor
The EMBO Journal

2nd Revision - Authors' Response 28 May 2011

Thank you very much for getting an additional referee to consider our manuscript. We are currently performing the first experiment requested by the reviewer and modifying the manuscript in response to the other points raised. I will send you the revised manuscript as soon as the experiment and replicates are completed.

Specific Responses:

1) We will perform this experiment and add it to the manuscript. Please note that, as explained in the response and revised Introduction, the activation of Src observed in the RPTP 245 expressing cells may have occurred either with or without phosphorylation of Src Tyr 419. Thus, this experiment can neither support nor contradict the direct demonstration (by the enolase phosphorylation assay), that RPTP 245 activates Src. Nonetheless, it will be interesting to know the status of autophosphorylation in this activated state.

2) The plasmid used for expressing RPTP 245 only contains the coding region from the human cDNA and not the downstream region that normally encodes D1 and D2. The siRNAs target this deleted region, which is present only in eRPTPα. Downregulation of eRPTPα and not RTPTα245 is clearly demonstrated in the immunoblots in Fig. 4b. However, we now realize that these points, which depend both on the locations of the siRNAs and the cloning primers, were not made clear in the text. We have added statements to Results, Materials and Methods, and the Table S3 legend to clarify this point. We think that these changes are adequate, and for space reasons think it best not to move Table S3 to the main text.

3) We acknowledge that it may appear surprising that transient siRNAs can suppress growth in soft agarose. However, this experiment is analogous to prior experiments (Zheng et al, 2008) demonstrating that either Src or RPTPα siRNAs inhibit the growth in soft agarose of tested human colon and ER breast cancer cell lines. Two possible explanations:
a) Our control experiments show that the siRNA suppression lasts for about four days in dividing cells in monolayer culture. However, the persistence of suppression may be longer in non dividing cells that lack anchorage possibly because the siRNAs are not diluted by cell division, because the depleted mRNAs are only slowly replaced in the arrested cells, or because the siRNAs are more slowly degraded in the arrested cells. (We not able to get a good measurement from cells that have been suspended in soft agarose to test this.)
b) The siRNAs may only delay and not completely suppress anchorage independent growth. The duration of the assay (21 d) is determined by signal/noise considerations. It takes almost the full 21 d to get a clear positive signal from the transformants. Therefore, even a transient retardation of growth will appear as a suppression of anchorage independent growth. (This is a familiar instance of examining an S shaped curve.)

4) We think it most likely that eRPTPα is inhibited by wedge insertion both in the eRPTPα homodimers (i.e., according to the Bilwes et al structure) and in the eRPTPα RPTP 245 heterodimer (i.e., as per Fig. S2). Thus, its activity is inhibited, and equal, in both cases. As stated in Results, the MBP assay was checked for linearity by using both 5 and 10 minute incubation times.

5) Grb2 overexpression is expected to induce multiple changes in signal transduction pathways For example, Skolnik et al (Science, 1993, 260:1953) showed that overexpression increases ERK tyrosine phosphorylation and activity, which presumably also activates downstream processes. Such generalized changes may affect RPTPα homodimerization, its association with Src in focal adhesion plaques or other locations, its (probably PKC mediated) phosphorylations at Sers 180 and 204, and/or its phosphorylation at Tyr789 independently of RPTP 245. Because of these multiple potential effects, we have no prediction as to the net effect of Grb2 overexpression on RPTP activity or on the ability of RPTP 245 to activate eRPTP . Moreover, since almost all pTyr789 is bound by Grb2 in normal cells it is not evident that the amount of available Grb2 is limiting or that changing its expression level would affect RPTP activity -either result could be explained within the

context of our model. Thus, we do not know how to interpret such experiments. A study of the effects of Grb2 overexpression on RPTPα Src mediated signaling would be interesting and would be a necessary precursor to studies of the effect of Grb2 overexpression on the effect of RPTPα245. However, this will be a challenging study that must monitor multiple parameters and include numerous controls. We do not believe it is appropriate for this paper.

Pre-acceptance Letter 01 June 2011

Thank you very much for submitting your revised version that I will be happy to accept eventually.
Before this, I do like to point out that we increasingly encourage our authors to be precise about statistical analyses. Running through your figures, I realized that in PTP-activity-assays (Fig.5 B versus D) you claim in both cases to represent standard deviations from n=2. As far as I understand, 5B represents to independent experiments, while n=2 would than reflect replicates of aliquots from the same IP?
Fig5D however would than only represent the latter (and thus reveal accurate pipetting instead of independent experimentation)?

I kindly ask you to specify for readers to be able to follow what had actually been done here.
I am very much looking forward to an amended doc-file that I am happy to upload into the system.

The editorial office will subsequently be in touch related to formal acceptance.

I would like to congratulate you on your study and remain with best regards.

Additional Correspondence 02 June 2011

I am very pleased to hear that the manuscript is close to acceptance.

Re Fig.5: The legend as written was misleading. I apologize.

Fig.5D represents average from two independent experiments both using IPs of the type shown in

Fig.5B. Therefore, the n=2 specification is correct. The legend has been revised and now reads:
(D) Equal portions of the anti-HA immunoprecipitates from two independent experiments like that shown in (C) and parallel immunoprecipitates from RPTPaHA-expressing cells (not shown) were used in a [32P]Tyr-MBP dephosphorylation assay as in (B). Relative amounts of released 32P are shown (s.d.'s;n=2).

We have added an Author contributions section following Acknowledgments.

Please note that the running title that appears on the web listing of the manuscript is incorrect (it refers to PTPa rather than RPTPa, the revised nomenclature).The running title listed on the title page of the manuscript is correct.The revised manuscript is attached.

Thank you very much for all efforts in reviewing this manuscript.

