**SPRY2 loss enhances ErbB trafficking and PI3K/AKT signaling to drive human and mouse prostate carcinogenesis**

Meiling Gao, Rachana Patel, Imran Ahmad, Janis Fleming, Joanne Edwards, Stuart McCracken, Kanagasabai Sahadevan, Morag Seywright, Jim Norman, Owen Sansom and Hing Leung

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**Transaction Report:**

(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 21 October 2011

Thank you for the submission of your manuscript "SPRY2 loss enhances ErbB trafficking and PI3K/AKT signaling to drive human and mouse prostate carcinogenesis" to EMBO Molecular Medicine and please accept my apologies for the delayed reply. We have now received the reports from the three referees whom we asked to review your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

Importantly, reviewer #2 highlights that the effect SPRY2 knockdown in PTEN-negative cells should be further explored with regards to EGFR expression as well as cell proliferation and invasion. Reviewer #3 points out that the clinical samples should be further investigated to strengthen the in vitro findings. In addition, reviewer #1 highlights that improved statistical analyses and a number of important controls should be included.

Of note, all reviewers highlight that the description of figures and experiments should be improved. Please also ensure that all data that are referred to in the manuscript are shown either in the main figures or the supporting information since EMBO Molecular Medicine does not allow references to 'data not shown'. We would also strongly encourage you to carefully edit the text and provide a schematic model of the investigated pathways as suggested by reviewer #3.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the time constraints outlined below.
However, we realize that addressing all of the referees' criticisms might require a lot of additional time and effort and be technically challenging. I am uncertain whether you will be able (or willing) to return a revised manuscript within the 3 months deadline and I would also understand your decision if you chose to rather seek rapid publication elsewhere at this stage.

Should you decide to embark on such a revision, revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions unless discussed differently with the editor. Should you find that the requested revisions are not feasible within the constraints outlined here and choose, therefore, to submit your paper elsewhere, we would welcome a message to this effect.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The studies use standard methodologies but they are novel in that for the first time this group is showing the role of Pten and Spry2 in prostate cancer. Although one study showed involvement of Pten in the anti-proliferative actions of Spry2 in cultured cells, this is the first time that this relationship is being investigated in prostate cancer using animal model and prostate cancer tissue microarrays. However, as indicated in my comments to the authors, they need to perform extensive revisions and incorporate additional experiments before this manuscript can be considered acceptable for publication in EMBO Mol Med.

Referee #1 (Other Remarks):

The manuscript by Gao et al., presents some interesting data suggesting an intricate relationship between loss of Spry2 and Pten in enhancing prostate carcinoma growth and invasiveness. The strengths of the manuscript include the inclusion of data from TMAs and correlation of findings with disease progression/longevity of patients to studies in prostate cancer cell lines that express Pten or are deficient in Pten expression. However, there are several major concerns that need to be addressed. One of the major underlying concerns that makes data interpretation harder is that the figure legends are very short missing important details such as times of treatment or conc. of drugs, etc. In some places the figure legends do not correspond with what is being shown.

Major Concerns:

Fig. 1: The bar graphs should show error bars and the number of samples quantified for each tumor grade should be indicated in the figure legend. Since these and other data from human prostate cancers rely on IHCs, some control for specificity of the Spry2 antibody and indication of exactly which antibody from what source was used is important.

Fig. 2: Panel A - how long were the cells treated with heregulin or EGF? What are the phospho-Her2 and phospho-EGFR specifically against - i.e. what Tyr residues on the receptors? Fig. 2C - the data from a number of experiments should be pooled and quantified to reflect the % of cell surface receptor that is endocytosed. Only then can the effect of Spry2 KD be assessed. Fig. 2D: What do "T" and "B" stand for? It appears that decreasing Spry2 in PC3 cells decreases EGFR levels. Again this should be quantified from more than one expt. Also does ectopic expression of Pten in PC3 cells overcome the decrease in EGFR when Spry2 is silenced?

Fig. 3: It is important that to tie Pten with Spry2, the authors perform expts similar to those in panels B and D in PC3 cells that have been transfected to express Pten. Likewise, the levels of Pten in DU145 cells {plus minus} Spry2 KD should be shown. Because Pten levels alone do not reflect the activity of Pten, blots with phospho-Pten Abs should also be performed. This is essential since in the paper cited by the authors (Edwin et al), Spry2 increased Pten content as well as activity by
stabilizing the protein. Likewise, when Pten is silenced, the authors need to monitor Spry2 content - is the increase in invasiveness due change in Spry2 levels? Figs. 3E and 3F are performed in DU145 cells, yet the fig legend states PC3 cells! Also, the fig. legend indicates invasion and the label on the Y axis of Fig. 3F is growth!!

Figure 4: Panel A: The quantification of data from one experiment or different exposures of the same experiment (Fig. S8) is not adequate. One needs to include error bars from more than one exp. Panel B: Does Ly294002 treatment alter Spry2 levels or Pten levels? Panel D: Need to show the same with and without EGF in parallel expts. Panel E: The stated difference in phospho-ERK (Pg. 9) does not seem to be corroborated by the single experiment - the authors should quantify data from different experiments.

Figure 5: What does SB compound do to EGFR phosphorylation on Tyr residues. This is important to be able to interpret data in panels C-E since no effects would be expected if the receptor was not activated. The authors should also show whether the SB compd alters EGF-induced changes in phospho-S1046/1047 - this will support the model shown at the bottom of the figure. Experiments like the ones shown in panel D with siRNA against p38 should also be performed to control for non-specific effects of the SB compd. Panel G: What phospho-sites of the EGFR are being probed? If phospho-Tyr is being monitored, then why would PI3K inhibition block this?

Figure 6: panels B needs to include Pten fl/- Spry+/+ and panel C should also show wt and Pten fl/- Spry +/- for complete interpretation.

Figure 7: The authors should check of PI3K inhibitor changes Spry2 levels.

Referee #2:

Summary:
The manuscript entitled "SPRY2 loss enhances ErbB trafficking and PI3K/AKT signaling to drive human and mouse prostate carcinogenesis" by Gao et al describes how SPRY2 functions synergistically with aberrant activation of the Akt pathway to drive prostate cancer and promote treatment resistance. The authors postulate that there is a positive feedback regulation of ErbB-PI3K/Akt. Loss of SPRY2 results in hyperactivation of Akt and enhances EGFR and Her2 internalization which is accompanied by an increase of cell proliferation and invasion in a PTEN dependent manner. This process involves p38 kinase mediated clatherin-Erb2 endocytosis.

Comment:
The effect of SPRY2 on Her2/EGFR trafficking is not novel and has been reported previously by Kim et al., 2007 and was not mentioned by the authors. Moreover Sprouty family members have been shown to negatively regulate MAPK and PI3K signaling via PTEN (Edwin et al., 2006) and PTEN has been already been shown to be necessary for SPRY2 mediated inhibition of cell proliferation (Edwin et al., 2006). Recently Faratian et al., 2011 showed that SPRY2 is associated with an increase in pathological grade, high Her2 expression and is a significant independent prognosis factor in breast cancer.

The description of figures is weak and lacks details making it difficult for the reviewer to understand. For example in Figure 2D it is unclear what "T" and "B" mean. There is no clarification of this either in the figure itself or the legend or text of the manuscript.

The authors report in the text that in BPH both basal and luminal cells express low to moderate expression of SPRY2, however no corresponding figure was integrated in the manuscript. In figure 1, the authors should change their legend from grade to Gleason score as this is more relevant clinically.

Data from figure 2A-B describe the effect of silencing SPRY2 on Her2 and Her3 phosphorylation. This figure is missing a required total Her2 and Her3 as a control for protein phosphorylation. This figures also require their downstream effectors to show that SPRY2 knockdown regulates Her2 and Her3 signaling pathways. Moreover, the effect of SPRY2 knockdown should also be assessed in PTEN negative cell lines.

Figure 2 The internalization of Her3 should be performed in the presence of Heregulin. In PTEN negative cells (PC-3), SPRY2 knockdown leads to EGFR degradation. Is this effect at the protein or mRNA levels? This is a very important and novel observation and this effect should not be ignored and should be addressed or at least discussed. This has clinical relevance since 70% of
prostate cancer patients have altered PTEN.
In the same line, SPRY2 knockdown inhibits proliferation and invasion in PC-3 cells while it increases proliferation and invasion in DU145 cells. These data point out that SPRY2 has the opposite effect on cells that are positive or negative to PTEN. To further confirm these observations, the authors need to use other cell lines that are positive and negative of PTEN to further support their data on cell proliferation and invasion.
Figure 4A and figure S7 should be swapped, moreover figure S7 lacks total protein expression (Akt and Erk).
The authors conclude that SPRY2 abolished EGF induced pAkt in early endosomes. However, the experiment was done in the absence of EGF treatment (figure 4D-E). This experiment should be done in the presence of EGF.
Is the effect of p38 kinase on EGFR internalization Akt dependent/PTEN?
One of the most established and well accepted Akt downstream effectors is phospho-S6Kinase. Does alteration of S6kinase affect EGFR and Her2 internalization?
The data from transgenic mice are interesting. The authors should include comparative studies between Ptenfl/+ and Ptenfl/+ Spryfl+/− in every panel in figure 6 to further convince the reviewer of the impact of SPY2 loss in Ptenflox background.
PI103 treated animals showed changes in the morphology of prostate tissue. How do the authors explain these changes? Is this effect specific to Ptenfl/+ Spryfl+/−?

Referee #3 (Comments on Novelty/Model System):
The major observation of this paper is that in the presence of PTEN, SPRY2 is a negative regulator of EGFR and HER2 whereas in the absence of PTEN it is not. This is a very important observation, because it explains the variations in HER2 that is seen in various patients with prostate cancer.

Referee #3 (Other Remarks):
In this manuscript, the authors study the effects of down-regulation of Sprouty-2 (SPRY2), a putative tumor suppressor, in prostate cancer. SPRY2 is known to inhibit receptor tyrosine kinases (RTK) signaling and the authors of this manuscript investigate its known interaction with ErbB2, a member of the epidermal growth factor receptor (EGFR) family. They previously showed in 2005 that SPRY2 is epigenetically silenced in 70% of primary prostate cancer, and others showed that SPRY2 plays a role in down-regulating RTK signaling. In this paper, they show data suggesting that high grade tumors expressed lower levels of SPRY2 compared to low grade ones and that SPRY2 levels correlated negatively with HER2.
Significantly, in vitro studies showed that the negative correlation was seen in the presence of PTEN whereas in the absence of PTEN, SPRY2 levels positively correlated with the levels of EGFR, HER2 and cell growth. This is the most important observation in the manuscript, and yet, the authors do not seem to be giving enough space to this observation. One would expect that in keeping with the in vitro results, the authors would stain for PTEN, SPRY2 and EGFR and statistically correlate SPRY2 and EGFR or HER2 in the presence or absence of PTEN but this was not done. Similarly, the authors failed to comprehensively provide an explanation for the various observations made in vitro, or even provide a schematic representation of the various pathways interact.
1. Figure 1: Data from 411 tumors and 137 non-tumor tissues were shown in this manuscript, yet there is no detailed information on who the patients are. Where were these tissues obtained? It is typical to present a table showing details about the patients - ages, Gleason scores, PSA at surgery, etc. Without this information, the data is not very meaningful.
2. The authors refer to data showing that "none of the other members of the ErbB receptors showed any association with patient outcome" (page 5, lines 10-11) and "We examined the activation of RTK including HER2, EGFR and HER3 in response to heregulin and EGF" (page 5, lines 31-32). Yet the data with EGFR or HER3 is not shown in either case. Please provide the relevant data, if only as a supplement.
3. There is no statistical correlation of high vs low SPRY2 and patient outcome. Figure 1B shows data only in low SPRY2. Please show what happens in high SPRY2 patients.
4. The correlation between SPRY2 and HER2 is shown in a single patient in Figure 1C. Please show statistical data using all the patients.
5. Figure 1A shows 50% reduction in the % of tumors expressing strong SPRY2 if the tumors are
high grade. Is there a difference in HER2 expression between those that expressed SPRY2 s those who did not?

6. Figures 1A 1-c: Please show high res pictures in the supplement.
7. Figure 2C: Please show both clones.
8. Use of another set of cell lines - such as LNCaP which lacks PTEN and CWR22 Rv1 which expresses PTEN will verify the data.

Detailed responses to the Editor’s comments and Reviewers’ reports

Importantly, reviewer #2 highlights that the effect SPRY2 knockdown in PTEN-negative cells should be further explored with regards to EGFR expression as well as cell proliferation and invasion. Reviewer #3 points out that the clinical samples should be further investigated to strengthen the in vitro findings. In addition, reviewer #1 highlights that improved statistical analyses and a number of important controls should be included.

Of note, all reviewers highlight that the description of figures and experiments should be improved. Please also ensure that all data that are referred to in the manuscript are shown either in the main figures or the supporting information since EMBO Molecular Medicine does not allow references to ‘data not shown’. We would also strongly encourage you to carefully edit the text and provide a schematic model of the investigated pathways as suggested by reviewer #3. EMBO Molecular Medicine

We appreciate the criticism regarding the description of figures and experiments very much. These are updated with the relevant information and we have also carefully edited the manuscript.

All data are now shown as either main figures or as supporting information. In addition, we have incorporated significant amount of new data as well as improved images from the original submission. New/modified data are highlighted with red panel label within the main figures. There are also substantial additions to the supplementary figures. Finally, we have designed a schematic model as suggested by Reviewer 3 (Figure 8).

Specific responses to reviewers' comments

Referee #1 (Comments on Novelty/Model System):

The studies use standard methodologies but they are novel in that for the first time this group is showing the role of Pten and Spry2 in prostate cancer. Although one study showed involvement of Pten in the anti-proliferative actions of Spry2 in cultured cells, this is the first time that this relationship is being investigated in prostate cancer using animal model and prostate cancer tissue microarrays. However, as indicated in my comments to the authors, they need to perform extensive revisions and incorporate additional experiments before this manuscript can be considered acceptable for publication in EMBO Mol Med.

We welcome this reviewer’s comment and have carried out significant additional experiments and revised the manuscript extensively. We believe that our report has significantly improved and are hopeful that it is now suitable for publication in EMBO Molecular Medicine.

Referee #1 (Other Remarks):

The manuscript by Gao et al., presents some interesting data suggesting an intricate relationship between loss of Spry2 and Pten in enhancing prostate carcinoma growth and invasiveness. The strengths of the manuscript include the inclusion of data from TMAs and correlation of findings with disease progression/longevity of patients to studies in prostate cancer cell lines that express Pten or are deficient in Pten expression. However, there are several major concerns that need to be
addressed. One of the major underlying concerns that makes data interpretation harder is that the figure legends are very short missing important details such as times of treatment or conc. of drugs, etc. In some places the figure legends do not correspond with what is being shown.

All figure legends are now carefully revised to include the relevant information. Oversights in the figure legends are now corrected.

Major Concerns:

Fig. 1: The bar graphs should show error bars and the number of samples quantified for each tumour grade should be indicated in the figure legend. Since these and other data from human prostate cancers rely on IHCs, some control for specificity of the Spry2 antibody and indication of exactly which antibody from what source was used is important.

Figure 1 is significantly revised with improved images. The tumour ‘grades’ are now described as Gleason grade and the numbers of cases for each category are provided in the figure legend. The bar graph shows the absolute percentage of cases satisfying a specific histoscore threshold, namely >200. In this analysis, we did not generate error bars.

Specific SPRY2 antibody from Abcam (ab50317) was optimised for our experiments. This antibody is a rabbit polyclonal antibody raised against the amino-terminal of SPRY2 protein. It reacts specifically with both human and mouse SPRY2 protein. Previously, using a similar in-house antibody, Fritzsche et al. immunostained for SPRY2 expression in prostate epithelial cells in human tissue sections (Fritzsche et al., Endocrine Related Cancer 13: 839-49, 2006). Besides our own work, the specific SPRY2 Abcam antibody (ab50317) has been well described for the detection of SPRY2 by Western blotting: human SPRY2 by Miraoui et al., Hum Mol Genet 19:1678-89, 2010 and murine SPRY2 by Hatley et al., Cancer Cell 18: 282-93, 2010. We have further optimised the use of this antibody in both clinical and murine samples. In Figure 6B, the intensity of SPRY2 immunoreactivities in the prostates directly reflected the status of SPRY2 genotype in the transgenic mouse models.

Fig. 2: Panel A - how long were the cells treated with heregulin or EGF? What are the phospho-Her2 and phospho-EGFR specifically against - i.e. what Tyr residues on the receptors? Fig. 2C - the data from a number of experiments should be pooled and quantified to reflect the % of cell surface receptor that is endocytosed. Only then can the effect of Spry2 KD be assessed. Fig. 2D: What do "T" and "B" stand for? It appears that decreasing Spry2 in PC3 cells decreases EGFR levels. Again this should be quantified from more than one exp. Also does ectopic expression of Pten in PC3 cells overcome the decrease in EGFR when Spry2 is silenced?

In Figure 2 panel A, cells were stimulated with heregulin (50 ng/ml) or EGF (20 ng/ml) for 15 mins. The phospho-specific antibodies against HER2 and EGFR recognise p-HER2 at Y877 and p-EGFR at Y1068, respectively.

We appreciate this reviewer’s comment very much and have carried out additional experiments and pooled data together for analysis. A new figure is now presented as Figure 2D showing pooled and quantified data to reflect the percentages of cell surface receptor that were endocytosed. In the original figure 2D, “T” represented the total surface EGFR, while “B” showed data from the blank control obtained from samples with reduced surface biotin without EGF stimulation, ie no internalisation. The figure legend is updated accordingly.

This is an interesting point that this reviewer made. Based on pooled quantitative data for EGFR protein levels in PC3 Nsi and SPRY2 KD clones from three independent experiments, we are able to confirm that decreasing Spry2 expression in PC3 cells indeed decreases EGFR levels (Figure S8). Furthermore, ectopic expression of PTEN in PC3 SPRY2 KD clones partially rescued EGFR expression at the protein level (Figure S9).

Fig. 3: It is important that to tie Pten with Spry2, the authors perform expts similar to those in panels B and D in PC3 cells that have been transfected to express Pten. Likewise, the levels of Pten in DU145 cells {plus minus} Spry2 KD should be shown. Because Pten levels alone do not reflect
the activity of Pten, blots with phospho-Pten Abs should also be performed. This is essential since in the paper cited by the authors (Edwin et al), Spry2 increased Pten content as well as activity by stabilizing the protein. Likewise, when Pten is silenced, the authors need to monitor Spry2 content - is the increase in invasiveness due to change in Spry2 levels? Figs. 3E and 3F are performed in DU145 cells, yet the fig legend states PC3 cells! Also, the fig. legend indicates invasion and the label on the Y axis of Fig. 3F is growth!!

Restoration of PTEN in PC3 cell lines resulted significant increase in proliferation in SPRY2 KD derived clones (Figure 3G). Of note, PC3 cells with transfected PTEN expression showed similar growth pattern when compared with the PTEN positive DU145 cells. Hence, these data are consistent with the notion that PTEN is required for SPRY2 lost mediated effects.

In the SPRY2 KD DU145 cells, the p-PTEN and total PTEN expression level was assessed. Suppressed SPRY2 expression did not change the levels of p-PTEN (s380) or total PTEN significantly. Hence, SPRY2 loss mediated EGFR signalling does not appear to depend on changes in p-PTEN or total PTEN levels (Figure S11).

On the other hand, suppression of PTEN expression by siRNA treatment did not influence SPRY2 expression: PTEN siRNA transfection in DU145 SPRY2 KD cells did not change SPRY2 expression with consistently low SPRY2 levels when compared to the control Nsi cells (Figure S10).

We apologise for the error in the figure legend. This has now been corrected.

Figure 4: Panel A: The quantification of data from one experiment or different exposures of the same experiment (Fig. S8) is not adequate. One needs to include error bars from more than one exp. Panel B: Does Ly294002 treatment alter Spry2 levels or Pten levels? Panel D: Need to show the same with and without EGF in parallel expts. Panel E: The stated difference in phospho-ERK (Pg. 9) does not seem to be corroborated by the single experiment - the authors should quantify data from different experiments.

Data in (the original) Figure 4 panel A was intended to highlight the most obvious changes observed, which were then further validated by relevant experiments. To avoid the impression of over-interpretation from these data, we have rearranged the figure to show the bar charts as supplementary data. The data for the less intense signal for p38 and MEK1/2 are now shown separately as they underwent a longer exposure time.

Representative Western blots for p-AKT and p-ERK1/2 are now shown as the updated Figure 4A. Previous publications using phospho-kinase arrays have also applied similar approach to ours, namely a single array analysis with duplicated spots for each kinase within the array, followed by validation focusing on the kinases of interest (Dewaele et al., Cancer Res; 70: 7304-14, 2010, Dunn et al., Oncogene; 30: 561-74, 2011).

Western blot analysis on the effects of LY294002 was presented in Figure 5G of the original submission. We now incorporate new data to show that LY294002 treatment did not significantly alter SPRY2 and PTEN expression.

For panel D, additional data from parallel experiments with and without EGF are now presented in the supplementary figure. As expected, in the absence of EGF stimulation, EGFRs are predominantly detected at the cell surface following Dynasore treatment (Figure S16).

For panel E, based on the suggestion from this reviewer, we have now formally quantified the p-ERK1/2 levels from three independent experiments. In this analysis, we found no significant difference in the p-ERK1/2 levels (p=0.29). We have therefore updated the description in the Results section accordingly.

Figure 5: What does SB compound do to EGFR phosphorylation on Tyr residues. This is important to be able to interpret data in panels C-E since no effects would be expected if the receptor was not activated. The authors should also show whether the SB compd alters EGF-induced changes in phospho-S1046/1047 - this will support the model shown at the bottom of the figure. Experiments like the ones shown in panel D with siRNA against p38 should also be performed to control for non-specific effects of the SB compd. Panel G: What phospho-sites of the EGFR are being probed? If
phospho-Tyr is being monitored, then why would PI3K inhibition block this?

The effect of SB compound was assessed, and inhibition of p38 resulted in reduced EGFR trafficking and hence the phosphorylation at tyrosine 1068. As the reviewer predicted, treatment with the SB compound to inhibit p38 function eliminated the differences observed between the Nsi and SPRY2 KD cells (following EGF stimulation), and impaired the activation of both S1046/7 and Y1068 within EGFR. Western blot showing the status of tyrosine-1068 blot is incorporated within Figure 5B.

In panel G, phosphor-S1046/1047 was probed for. The data shows that LY compound decreased the activity of p38 and hence reduced the EGFR phosphorylation. Overall, disrupting the positive feedback loop depicted in Figure 5H has a significant impact on activation of both S1046/7 and Y1068, thus supportive the functional importance of this SPRY2 loss mediated pathway.

Figure 6: panels B needs to include Pten fl/+ Spry+/+ and panel C should also show wt and Pten fl/- Spry +/+ for complete interpretation.

We appreciate this point and have updated the figures accordingly. Overall, interpretation of the completed data supports our proposed model.

Figure 7: The authors should check of PI3K inhibitor changes Spry2 levels.

To assess the level of SPRY2 expression in the murine prostates, we carried out immunohistochemistry experiment using the optimised specific SPRY2 antibody. When compared to vehicle treated animals, PI3K inhibitor treatment did not alter SPRY2 immuno-reactivity in the prostate. Representative images are shown (Figure S21).

Referee #2:

Summary: The manuscript entitled "SPRY2 loss enhances ErbB trafficking and PI3K/AKT signalling to drive human and mouse prostate carcinogenesis" by Gao et al describes how SPRY2 functions synergistically with aberrant activation of the Akt pathway to drive prostate cancer and promote treatment resistance. The authors postulate that there is a positive feedback regulation of ErbB-PI3K/Akt. Loss of SPRY2 results in hyperactivation of Akt and enhances EGFR and Her2 internalization, which is accompanied by an increase of cell proliferation and invasion in a PTEN dependent manner. This process involves p38 kinase mediated clatherin-Erb2 endocytosis.

Comment: The effect of SPRY2 on Her2/EGFR trafficking is not novel and has been reported previously by Kim et al., 2007 and was not mentioned by the authors. Moreover Sprouty family members have been shown to negatively regulate MAPK and PI3K signalling via PTEN (Edwin et al., 2006) and PTEN has been already shown to be necessary for SPRY2 mediated inhibition of cell proliferation (Edwin et al., 2006). Recently Faratian et al., 2011 showed that SPRY2 is associated with an increase in pathological grade, high Her2 expression and is a significant independent prognosis factor in breast cancer.

We thank this reviewer’s comment and insightful references. Within the initial submission, we apologise for omitting the Kim et al 2007 citation. We are very surprised with this oversight, which probably happened due to inadvertent deletion/editing. We have now included this relevant report in the Introduction section of our manuscript. Nonetheless, it is worth pointing out that the focus of their work was not on the functional impact of SPRY2 loss and potential effects on receptor tyrosine kinase function. Kim et al. (2007) reported EGFR trafficking mediated by SPRY2 function, but the interaction between ERGR/HER system and SPRY2 loss was not examined. Hence, our report provides timely and significant discovery on inactivation of SPRY2 in carcinogenesis and its
interaction with RTK system in a PTEN dependent manner using relevant resources for the in vitro, in vivo and clinical context.

The findings from Faratian et al 2011 are relevant and we have updated our manuscript and included this reference accordingly (page 3).

The description of figures is weak and lacks details making it difficult for the reviewer to understand. For example in Figure 2D it is unclear what "T" and "B" mean. There is no clarification of this either in the figure itself or the legend or text of the manuscript.

We appreciate the comment from this reviewer and apologise for the omission. The figure legends are updated and expanded. Mistakes are now corrected.

The authors report in the text that in BPH both basal and luminal cells express low to moderate expression of SPRY2, however no corresponding figure was integrated in the manuscript. In figure 1, the authors should change their legend from grade to Gleason score, as this is more relevant clinically.

A representative image for BPH showing moderate level of SPRY2 immunoreactivity is now included (Figure S1). The legend for Figure 1 is now updated as requested using Gleason grades.

Data from figure 2A-B describe the effect of silencing SPRY2 on Her2 and Her3 phosphorylation. This figure is missing a required total Her2 and Her3 as a control for protein phosphorylation. This figures also require their downstream effectors to show that SPRY2 knockdown regulates Her2 and Her3 signalling pathways. Moreover, the effect of SPRY2 knockdown should also be assessed in PTEN negative cell lines.

We agree with the reviewer. The total HER2 and HER3 as controls are now added in Figure 2A and B. In addition, the level of p-AKT was further assessed as a downstream ‘readout’ of HER signalling.

Figure 2- The internalization of Her3 should be performed in the presence of Heregulin.

We appreciate the reasoning for this request. However, since we did not observe any meaningful activation of HER3 or any significant effects following heregulin stimulation (Figure 2A), we have not specifically studied internalisation of HER3 following heregulin treatment. Based on these considerations and the fact that the only significant observations were made following EGF stimulation, we have focused our study on EGFR and HER2.

In PTEN negative cells (PC-3), SPRY2 knockdown leads to EGFR degradation. Is this effect at the protein or mRNA levels? This is a very important and novel observation and this effect should not be ignored and should be addressed or at least discussed. This has clinical relevance since 70% of prostate cancer patients have altered PTEN.

We completely agree with this reviewer’s comment about the relationship between SPRY2 KD and EGFR degradation (Figure 2E). EGFR expression at the mRNA level was therefore assessed using Q-RT-PCR in PC3 SPRY2 KD and Nsi control cells. EGFR mRNA level in PC3 SPRY2 KD cells was significant reduced when compared to the corresponding Nsi control cells (Figure 2E). Hence, our data suggest that the simultaneous loss of PTEN and SPRY2 results in reduced EGFR expression due to a reduction at its transcript level. This was further confirmed at the protein level by quantitative analysis (Figure S8).

In the same line, SPRY2 knockdown inhibits proliferation and invasion in PC-3 cells while it increases proliferation and invasion in DU145 cells. These data point out that SPRY2 has the opposite effect on cells that are positive or negative to PTEN. To further confirm these observations, the authors need to use other cell lines that are positive and negative of PTEN to further support their data on cell proliferation and invasion.
In SPRY2 KD PC3 cells, we observed no induction of proliferation upon EGF stimulation when compared to the control Nsi cells. This contrasts with the significant induction of proliferation in the PTEN proficient DU145 cells and the PC3 cells with transfected PTEN expression. To further test the relationship of PTEN and SPRY2 status in the regulation of proliferation, LNCaP cells, which is a human prostate cancer cell line deficient for both PTEN and SPRY2 expression, were studied. In the absence of PTEN expression, transfected SPRY2 expression did not influence cell proliferation nor p-AKT activation (Figure S12), thus recapitulating data from PC3 cells. We then investigated 22Rv1 cells (PTEN and SPRY2 positive). In 22Rv1 cells, similar to DU145 cells, stable loss of SPRY2 significantly enhanced cell growth as well as upregulated EGFR/HER2 phosphorylation along with PI3K/AKT activation as their downstream signalling partners (Figure S13).

Figure 4A and figure S7 should be swapped, moreover figure S7 lacks total protein expression (Akt and Erk).

Figures 4A and S7 are now swapped as requested. Loading control with total AKT and ERK1/2 are now included in the western blots.

The authors conclude that SPRY2 abolished EGF induced pAkt in early endosomes. However, the experiment was done in the absence of EGF treatment (figure 4D-E). This experiment should be done in the presence of EGF.

We apologise for the confusion with these two figure panels. These experiments were indeed performed in the presence of EGF stimulation. The figure legends are now updated to provide the relevant information.

Is the effect of p38 kinase on EGFR internalization Akt dependent/PTEN?

One of the most established and well accepted Akt downstream effectors is phospho-S6Kinase. Does alteration of S6kinase affect EGFR and Her2 internalization?

This is an interesting point that the reviewer made. Indeed p-S6K is one of several downstream effectors for AKT mediated signalling. Using Western blot analysis, we observed no difference in p-S6K level between SPRY2 KD and control Nsi cells following EGF stimulation. Hence, S6K is unlikely to play a key role in our observations (Figure S11). Besides S6K, other AKT substrates are well described: p-AKT can specifically phosphorylate its substrates at the Ser/Thr site within a conserved motif characterised by Arg at positions -5 and -3 (Alessi et al., FEBS letters; 399: 333-8, 1996). Hence we assessed p-AKT substrates in SPRY2 KD and Nsi control cells in the presence of EGF. The result indicates that a subset of AKT substrates were differentially activated due to SPRY2 loss upon EGF stimulation (see below).

Figure showing multiple substrates for p-AKT. Red Arrows highlight significant differences observed between SPRY2 KD and Nsi control cells. Western blot analysis was performed with specific antibody recognising the p-Akt substrate (RXXS/T) (Cell Signaling, 9614).

The data from transgenic mice are interesting. The authors should include comparative studies between Ptenfl/+ and Ptenfl/+ Spryfl+/- in every panel in figure 6 to further convince the reviewer of the impact of SPY2 loss in Ptenfllox background.

This is a very good point and the relevant images are now included in Figure 6 to provide a more comprehensive dataset.
PI103 treated animals showed changes in the morphology of prostate tissue. How do the authors explain these changes? Is this effect specific to Ptenfl/+ Spryfl+/−?

The in vivo experiment using PI103 treatment represents our proof of principle experiment aimed at testing our working hypothesis for a role of p-AKT in prostate tumorigenesis in the presence of reduced SPRY2 expression. We confirmed that PI103 did indeed drastically suppressed prostate tumour growth. Further work will be required to fully investigate the underlying mechanism of this effect and also the potential issue of treatment resistant disease. Our early data have suggested that induction of apoptosis may at least play a part in tumour regression. Our current interpretation of the change in morphology of the prostatic tissue reflects tumour regression but further detailed investigation with our in vivo mouse prostate model is required to fully address its molecular basis.

Referee #3 (Comments on Novelty/Model System):

The major observation of this paper is that in the presence of PTEN, SPRY2 is a negative regulator of EGFR and HER2 whereas in the absence of PTEN it is not. This is a very important observation, because it explains the variations in HER2 that is seen in various patients with prostate cancer.

Referee #3 (Other Remarks):

In this manuscript, the authors study the effects of down-regulation of Sprouty-2 (SPRY2), a putative tumour suppressor, in prostate cancer. SPRY2 is known to inhibit receptor tyrosine kinases (RTK) signaling and the authors of this manuscript investigate its known interaction with ErbB2, a member of the epidermal growth factor receptor (EGFR) family. They previously showed in 2005 that SPRY2 is epigenetically silenced in 70% of primary prostate cancer, and others showed that SPRY2 plays a role in down-regulating RTK signaling. In this paper, they show data suggesting that high grade tumours expressed lower levels of SPRY2 compared to low grade ones and that SPRY2 levels correlated negatively with HER2. Significantly, in vitro studies showed that the negative correlation was seen in the presence of PTEN whereas in the absence of PTEN, SPRY2 levels positively correlated with the levels of EGFR, HER2 and cell growth. This is the most important observation in the manuscript, and yet, the authors do not seem to be giving enough space to this observation. One would expect that in keeping with the in vitro results, the authors would stain for PTEN, SPRY2 and EGFR and statistically correlate SPRY2 and EGFR or HER2 in the presence or absence of PTEN but this was not done. Similarly, the authors failed to comprehensively provide an explanation for the various observations made in vitro, or even provide a schematic representation of the various pathways interact.

We thank Reviewer 3 for the supportive and insightful comments. Correlation analysis from our clinical cohort between the expression status of HER2 and SPRY2 as well as for p-AKT and p-ERK is included in Table 1.

Figure 8 has been designed to show a schematic representation of the various pathways analysed in the context of SPRY2 and HER mediated signalling.

1. Figure 1: Data from 411 tumours and 137 non-tumour tissues were shown in this manuscript, yet there is no detailed information on who the patients are. Where were these tissues obtained? It is typical to present a table showing details about the patients - ages, Gleason scores, PSA at surgery, etc. Without this information, the data is not very meaningful.

   We apologise for the omission of important clinical information. The relevant information are now included as a supplementary table to the report (Supplementary Table I).

2. The authors refer to data showing that "none of the other members of the ErbB receptors showed any association with patient outcome" (page 5, lines 10-11) and "We examined the activation of RTK including HER2, EGFR and HER3 in response to heregulin and EGF" (page 5, lines 31-32).
Yet the data with EGFR or HER3 is not shown in either case. Please provide the relevant data, if only as a supplement.

The relevant information is now provided within supplementary figure S3.

3. There is no statistical correlation of high vs low SPRY2 and patient outcome. Figure 1B shows data only in low SPRY2. Please show what happens in high SPRY2 patients.

The survival outcome for patient with SPRY2 expressing prostate cancer is now included as supplementary data (Figure S3). This shows that there is no association of patient survival based on HER2 stratification.

4. The correlation between SPRY2 and HER2 is shown in a single patient in Figure 1C. Please show statistical data using all the patients.

The reviewer is correct that representative images of SPRY2 and HER2 immunoreactivity from a single patient were presented in Figure 1C. We have corrected the figure legend and are presenting the overall summary data in Table 1.

5. Figure 1A shows 50% reduction in the % of tumors expressing strong SPRY2 if the tumors are high grade. Is there a difference in HER2 expression between those that expressed SPRY2 s those who did not?

The reviewer is making an interesting point. While SPRY2 expression inversely correlates with tumour grade (Gleason score), HER2 expression increases with increasing tumour grade. This information is now incorporated in the bar chart within Figure 1A.

6. Figures 1A 1-c: Please show high res pictures in the supplement.

We have improved the images and updated panel A.

7. Figure 2C: Please show both clones.

Pooled data from both cell clones are now included (Figure 2D).

8. Use of another set of cell lines - such as LNCaP which lacks PTEN and CWR22 Rv1 which expresses PTEN will verify the data.

There is a similar comment made by Reviewer 2. We have now studied both LNCaP and 22Rv1 cells, and the additional data supports our working hypothesis.

In SPRY2 KD PC3 cells, we observed no induction of proliferation upon EGF stimulation when compared to the control Nsi cells. This contrasts with the significant induction of proliferation in the PTEN proficient DU145 cells and the PC3 cells with transfected PTEN expression. To further test the relationship of PTEN and SPRY2 status in the regulation of proliferation, LNCaP cells, which is a human prostate cancer cell line deficient for both PTEN and SPRY2 expression, were studied. In the absence of PTEN expression, transfected SPRY2 expression did not influence cell proliferation nor p-AKT activation (Figure S12), thus recapitulating data from PC3 cells. We then investigated 22Rv1 cells (PTEN and SPRY2 positive). In 22Rv1 cells, similar to DU145 cells, stable loss of SPRY2 significantly enhanced cell growth as well as upregulated EGFR/HER2 phosphorylation along with PI3K/AKT activation as their downstream signalling partners (Figure S13).
As you will see, the reviewers acknowledge that the manuscript was improved during revision and they remain interested in the study. However, while reviewer #3 indicates that the manuscript is suitable for publication after including additional information in Table 1, reviewers #1 and #2 still raise significant concerns about the conclusiveness of the results. Since we do acknowledge the potential interest of your findings, we would be willing to consider a revised manuscript with the understanding that the referee concerns must be convincingly and conclusively addressed.

Importantly, the reviewers point out that crucial controls are still missing and have to be performed to conclusively support your findings.

On a more editorial note, please note that the manuscript must include an ethics statement regarding the compliance of experimental procedures using animals with relevant guidelines and regulations (please see excerpt from the Guide to Authors below). In addition, please see below for information regarding EMBO Molecular Medicine guidelines for statistical analysis of data. Please mention the actual p value in each case.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

A LARGE NUMBER OF DATA DO NOT HAVE CONTROLS WITHOUT EGF - THUS THE DATA CANNOT BE INTERPRETED ESPECIALLY WHEN DRUGS INHIBIT CONTROL CELL GROWTH OR INVASIVENESS BELOW THOSE IN CONTROL CELLS.

Referee #1 (Other Remarks):

This is a revised manuscript by Gao et al. in which the authors claim that the previously raised concerns have been addressed. Unfortunately, however, a large number of the previous concerns still remain. Problems are noted for a large number of primary and supplemental data. They are enumerated below.

1) Fig. S1 - cannot be interpreted - there is no comparison next to it. Thus, the authors claim on P. 5, line 9 is uninterpretable.
2) Fig. S3 - what about the opposite correlation of EGFR (panel a) and ErbB4 (panel c)?
3) Fig. 3: All growth and invasion assays need data without EGF - how does one know how much of the growth stimulation or invasiveness is due to EGF? The {plus minus} EGF data were requested last time! Minus EGF data for Fig. S12, S13, S17, S18 and other figs dealing with growth and invasion e.g. Fig. 4B, 4C, 4E, 5C, 5D, 5E, etc. are still missing!
4) The figure legends (primary and supplemental data) are still incomplete - do not inform the reader about time of EGF treatment, etc. This was also asked last time!
5) Fig. 6 and 7: Previously requested controls are still missing - will not repeat them here.
6) Where are data referred to on Pg. 8, lines 1 and 2?
7) Fig. S12 - no effect of EGF - 0 min and 15 min are the same!
8) Fig. S14 - changes are modest and cannot be interpreted without minus EGF data
9) Fig. 4A and other westerns in other figs - need bar graphs with error bars from more than one experiment - this was also requested last time.
10) The paper by Wee et al does not deal with Spry2 as alluded to on Pg. 8, last line.
11) Pg. 8, line 26 - incorrect - Fig. 2A shows activation of pAKT by heregulin
12) Pg. 11, line 20 - incorrect - Spry2 expression seems increased in Fig. S21
13) Figs. S14 and S15 - where are data without EGF - i.e. are changes EGF- specific? Also what are the blue and green rectangles - not explained in fig legend.

In sum, without the right controls, a lot of the data are uninterpretable and therefore there are major deficiencies with this manuscript.

Referee #2:

In this re-submission, the authors did an extensive revision and added new data and respond to all the comments. However one of the most important observations is that PTEN status determines the faith of EGFR/Her-2 and cell growth and this is independent of SPRY2 (opposite result between effect of SPRY2 knockdown in DU145 compared to PC-3). The authors didn't elucidate how this is regulated and the reviewer felt thirsty to know more about this effect and disappointed that was not even discussed in the discussion part.
The reviewer also observed that some data still missing controls or downstream effectors (this is a high impact journal):
- Figure 2, panel B: needs downstream effectors like pAkt/T-Akt
- Figure 4, panel A: expression of total proteins (Akt, Erk)
- Figure 5, panel B: expression of total Akt, p-p38 kinase/T-p38 kinase
- Figure 5, panel G: expression of total p38 kinase

Referee #3 (Comments on Novelty/Model System):
The novelty of the study is high as there are only 6 papers in Pubmed on SPRY2 in prostate cancer, and certainly none in conjunction with HER2 or PTEN or the PI3K/Akt pathway. Therefore the studies proposed are undoubtedly novel. The clinical impact is not immediate because the authors have not defined a method of regulating SPRY2 as of yet.

Referee #3 (Other Remarks):
The authors have made substantial changes to the manuscript since last submission and have answered all of the reviewers questions. In particular, the addition of the extensive supplementary data detailing patients profiles certainly add to the appeal of the manuscript.

However, Table 1 is not very clear. Please include the intensity range of each parameter used to arrive at the Pearson's correlation. For example, what is the min max and median staining intensity for each of those parameters?

2nd Revision - Authors' Response 06 April 2012

Reviewer's comments

Referee #1 (Comments on Novelty/Model System):

*A LARGE NUMBER OF DATA DO NOT HAVE CONTROLS WITHOUT EGF - THUS THE DATA CANNOT BE INTERPRETED ESPECIALLY WHEN DRUGS INHIBIT CONTROL CELL GROWTH OR INVASIVENESS BELOW THOSE IN CONTROL CELLS.*

We appreciate this concern by referee 1 and have now carefully revised each figure to ensure adequate information on experimental design and data analysis are provided throughout the report.

Referee #1 (Other Remarks):
This is a revised manuscript by Gao et al. in which the authors claim that the previously raised concerns have been addressed. Unfortunately, however, a large number of the previous concerns still remain. Problems are noted for a large number of primary and supplemental data. They are enumerated below.

1) **Fig. S1** - cannot be interpreted - there is no comparison next to it. Thus, the authors claim on P. 5, line 9 is uninterpretable.

Figure S1 now shows representative immunohistochemistry images of SPRY2 staining at low and moderate intensities in benign prostatic hyperplasia (BPH) samples.

2) **Fig. S3** - what about the opposite correlation of EGFR (panel a) and ErbB4 (panel c)?

Panel e now shows analysis of the prognostic value of EGFR, HER2, HER3 and HER4 expression among SPRY2 expressing tumours in the human PC TMA2. Cases were segregated according to respective expression of EGFR family members. Among SPRY2 positive tumours, the expression status of members of the EGFR family was not prognostic in Kaplan-Meier analysis.

3) **Fig. 3**: All growth and invasion assays need data without EGF - how does one know how much of the growth stimulation or invasiveness is due to EGF? The {plus minus} EGF data were requested last time! Minus EGF data for Fig. S12, S13, S17, S18 and other figs dealing with growth and invasion e.g. Fig. 4B, 4C, 4E, 5C, 5D, 5E, etc. are still missing!

The reviewer is quite correct that the relevant controls are required. All of our experiments were carried out with the correct controls in the first instance. We apologize that details for the experimental design and data analysis were not made clearer before. We have revised the figure legends accordingly to make this clear to the reader.

All of the growth assays were indeed performed with the relevant serum free condition (ie without EGF) as control. As an illustration, we have highlighted the background of the details as below using Figure 3A as an example:

**Figure A**

![Figure A](image)

In this submitted format, the data has incorporated information obtained from serum free condition of each clone as a reference. The percentage increase in cell growth was calculated from (and normalized against) respective data from the serum free control for each of the cell clone (as illustrated in Figure B below, from which Figure A was derived from).

Thus, the respective percentage increase in EGF-induced growth illustrated in the Y-axis is appropriately controlled with the relevant control (namely Nsi and SPRY2 KD cells respectively).

**Figure B**
We apologise for not making this clear in previous submission. We have carefully clarified this issue of control used as described above in the methodology section as well as the legend of individual figures.

Data obtained by the invasion assay were obtained using EGF as a chemoattractant. Comparison was made between different derived cell clones from isogenic cell models. Without EGF present as a chemoattractant, this particular assay system is not informative as shown by data below when PC3 or DU145 cells were studied in the invasion chamber without the presence of EGF to support cellular invasion. There are typically up to 3 cells per field for each cell clones analysed.

Figure showing the lack of significant cell invasion in the absence of EGF as a chemoattractant. SF significant serum free condition; PC3 CL1 and CL10 (clone 3 and 10); DU145 CL13 and CL61 (clone 13 and 61).

4) The figure legends (primary and supplemental data) are still incomplete - do not inform the reader about time of EGF treatment, etc. This was also asked last time!
The figure legends both main and supplementary are extensively revised to improve their contents and clarity to the readers.

5) Fig. 6 and 7: Previously requested controls are still missing - will not repeat them here.

This is what reviewer 1 wrote in the last round of review: ‘Figure 6: panels B needs to include Pten fl/− Spry+/+ and panel C should also show wt and Pten fl− Spry +/+ for complete interpretation.’ We have included wt, Pten fl/+ Spry +/+ and Pten fl/+ Spry +/+− for comparison. Our data clearly showed the significance of synergistic interactions between Pten haploinsufficiency and Spry2 inactivation. To clarify the various panels, we have updated the labels for the genotypes Pten fl/+ to Pten fl/+ Spry +/+ to make it easier for the reader to compare against the data from wt and Pten fl/+ Spry +/+−. Critically, the prostates in Pten fl/+ Spry +/+− mice showed convincing tumour progression while those in Pten fl/+ Spry +/+ (ie pten haploinsufficient alone) only showed evidence of high grade prostatic intra-epithelial neoplasia but not full transformation. Furthermore, mice harbouring a Pten fl/fl genotype will be prone to develop Pten null senescent phenotype which was not a principle research focus in this report.

6) Where are data referred to on Pg. 8, lines 1 and 2?

This statement refers to Figure 3B. We have now updated the text.

7) Fig. S12 - no effect of EGF - 0 min and 15 min are the same!

The Figure S12 indeed shows no significant difference in cell growth upon EGF stimulation. This supports our hypothesis: In PTEN null LNCaP cells, loss of SPRY2 does not influence cell growth, similar to that observed for PC3 cells earlier. Interestingly, LNCaP cells intrinsically have high level of p-AKT even in serum free condition, and the loss of SPRY2 did not activate AKT further upon EGF stimulation. Activation of p-ERK1/2 confirmed the biological effects of EGF treatment.

8) Fig. S14 - changes are modest and cannot be interpreted without minus EGF data

Due to the limited experiments carried out using phosphor-kinase array, all implicated kinases were independently validated in the presence and absence of EGF treatment (see figure 4A and 5A).

9) Fig. 4A and other westerns in other figs - need bar graphs with error bars from more than one expt - this was also requested last time.

As requested, we have included formal quantitation of multiple western blots from at least three independent experiments. Figure 4A now incorporate a bar graph for p-AKT levels for DU145 NSi and CL61 cells. In addition, we have obtained data from at least three independent experiments and presented them as averages normalized to the respective unstimulated NSi isogenic cells. Western blots were quantified using Image J and the pixel intensities were normalised to Nsi control cells maintained in serum free condition. The relative ratios and representative western blots were shown in Figure 2 (A, B), 4 (A, E), 5A and 7C as well as S9, S11 and S21.

10) The paper by Wee et al does not deal with Spry2 as alluded to on Pg. 8, last line.

The referee is absolutely correct. This particular reference reported that PI3K pathway activation strongly influences the sensitivity of RAS mutant cells to MEK inhibitors, and argues for the clinical testing of combination MEK and PI3K targeted therapies. This error must have happened during the editing process and we apologise for the oversight. The text is now corrected. As the main point of this reference is distinct from our report, it is now removed.

11) Pg. 8, line 26 - incorrect - Fig. 2A shows activation of pAKT by heregulin

We agree with the reviewer, heregulin does activate p-AKT when compared to serum free condition.
However, Nsi control cells and SPRY2 KD cells have the same level of AKT activation. Thus loss of SPRY2 does not affect AKT phosphorylation level in SPRY2 KD cells when compared to Nsi control cells. We have updated the manuscript to explain more clearly.

12) Pg. 11, line 20 - incorrect - Spry2 expression seems increased in Fig. S21

We appreciate the reviewers concern, and we have included new representative figure, and in addition western analysis of the tissue is now added to the Figure S21. The quantification of the western showed that there is no difference in SPRY2 expression between vehicle and PI103 treated prostate tissue samples.

13) Figs. S14 and S15 - where are data without EGF - i.e. are changes EGF- specific? Also what are the blue and green rectangles - not explained in fig legend.

Positive and negative controls were labelled with blue boxes. Green boxes signify the targets for phosphorylation of interest and were identified by the number label as shown in the bar chart. The experiment using Human Phospho-kinase Antibody Array blot was done with EGF stimulation alone. Our focus was to study the difference between control NSi and SPRY2 KD (CL61) cells in order to examine the impact of SPRY2 function in EGF mediated signalling. All of the key candidate targets of interest were further validated in independent experiments using Western blotting with EGF stimulation and controlled with the absence of EGF stimulation.

Referee #2:

In this re-submission, the authors did an extensive revision and added new data and respond to all the comments. However one of the most important observations is that PTEN status determines the faith of EGFR/Her-2 and cell growth and this is independent of SPRY2 (opposite result between effect of SPRY2 knockdown in DU145 compared to PC-3). The authors didn't elucidate how this is regulated and the reviewer felt thirsty to know more about this effect and disappointed that was not even discussed in the discussion part.

The following section is now added to the discussion to further discuss our findings as suggested by reviewer 2.

Consistently with our findings, Chandralapaty et al (2011) also reported the enhanced expression of growth factor receptors (such as EGFR and HER2) upon AKT inhibition. Furthermore, Carver et al (2011) have previously shown that PTEN deficient prostate tumours and prostate cancer cells have decreased HER2/3 expression. Hence, besides PTEN, our finding that additional loss of another negative regulator, namely SPRY2, also decreased the expression of EGFR and HER2. Taken together, this suggests that expression of growth factor receptors is dependent on presence of negative regulators, and highlights the importance of feedback inhibition between RTK signalling cascades and growth factor receptor expression. The SPRY2 KD PC3 cells (CL10), show increased ERK activation. Hence, it is plausible that this activated ERK may be involved in decreased EGFR and HER2 expression via feedback inhibition. However, the exact mechanism by which the negative regulators (PTEN and SPRY2) regulate the expression of growth factor receptors needs further elucidation.


The reviewer also observed that some data still missing controls or downstream effectors (this is a high impact journal):
- Figure 2, panel B: needs downstream effectors like pAkt/T-Akt

We appreciate the reviewer’s suggestion, and the downstream pAKT and AKT blots are now added.

- Figure 4, panel A: expression of total proteins (Akt, Erk)

Total AKT and ERK blots are now added in Figure 4A.

- Figure 5, panel B: expression of total Akt, p-p38 kinase/T-p38 kinase

Total and p-p38 blots are now added to Figure 5B. While the p38 inhibitor SB203580 disrupts the catalytic activity of p38 by binding to the ATP-binding pocket, it does not directly alter the phosphorylation level of p38. SB203580 did however disrupt the feedback loop of EGFR trafficking and its downstream AKT signalling, therefore resulting in a reduction in the phosphorylation of p38. The downstream effect of SB203580 on p38 directly was assessed using ATF2, a substrate ATF2 (Figure S19).

- Figure 5, panel G: expression of total p38 kinase

The total p38 blot is now added to the Figure 5G.

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The novelty of the study is high as there are only 6 papers in Pubmed on SPRY2 in prostate cancer, and certainly none in conjunction with HER2 or PTEN or the PI3K/Akt pathway. Therefore the studies proposed are undoubtedly novel. The clinical impact is not immediate because the authors have not defined a method of regulating SPRY2 as of yet.

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The authors have made substantial changes to the manuscript since last submission and have answered all of the reviewers questions. In particular, the addition of the extensive supplementary data detailing patients profiles certainly add to the appeal of the manuscript.

However, Table 1 is not very clear. Please include the intensity range of each parameter used to arrive at the Pearsons correlation. For example, what is the min max and median staining intensity for each of those parameters?

Table 1 is revised to include the exact p values. The details of histoscores (range and median) for each of the parameters are now included.

Other editorial points:

An ethics statement regarding the compliance of experimental procedures using animals is now included.

All exact p values are now added in the figure legends, and in the figures * signifies p value <0.05 and ** signifies p value <0.01. The numbers of independent experiments are detailed in figure legends.