Author's response to reviews

Title: The Cyclin-Like Protein Spy1/RINGO Promotes Mammary Transformation and is Elevated in Human Breast Cancer

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Title: "The Cyclin-Like Protein Spy1/RINGO Promotes Mammary Transformation and is Elevated in Human Breast Cancer"

Running Title: "Oncogenic Properties of Spy1"

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We thank the editor, the executive editor and both reviewers for their valuable suggestions and favorable review of our article. We have made several edits as suggested by the reviewers and have provided discussion and rationale for the experiments that were not included. The specifics of this are addressed below.

Reviewer Tiffany Katz

1. We have added in the following discussion regarding the rationale for the doses that were selected.

“It was noted that while protein levels and subsequent colony formation increased proportionately with the amount of cDNA transfected, colony formation was not simply a reflection of total amount of Spy1 protein (Fig. 1A&B). Using approximately half the amount of transfected DNA for Spy1-TST resulted in a statistically significant reduction of overall protein levels, but at least a 2-fold increase in colony formation with high statistical significance (compare 30µg Spy1-WT vs. 15µg Spy1-TST). This suggests the possibility that threshold levels or stabilization of the Spy1 protein triggers a unique mechanism that may contribute toward Spy1-mediated tumorigenesis.”

- For the experiments using constant amount of DNA we added to the materials and methods “In brief, for experiments using a fixed amount of DNA per construct 10 µg of DNA was mixed...” Further on page 11 “The significant increase in colonies seen with the Spy1-TST mutant over Spy1-WT supports that the G2/M degradation mechanism previously described [11] may provide a protective barrier against this potentially oncogenic pathway. ... To test this hypothesis, soft agar assays were conducted using similar protein levels of either Spy1-WT or Spy1-TST, along with activated Ras (Ras-V12) as a positive control and empty pCS3 as a negative control (Fig. 1C)...”

- Given that dose and protein levels are the important discussion in this panel we carried out the errors between separate experiments and overall relative statistics as was suggested (Figure 1B). We also further carried out the stats suggested when we kept the transfection constant between constructs (Figure. 1E). This did strengthen the point regarding the significant differences in colony formation.

2. Additional experiments were not performed to obtain statistical information for the
figures suggested for the following reasons:

- Figure 4E is a control to ensure that Cdk1 and Cdk2 DN vectors were working appropriately – we have moved this panel into the supplementary material to avoid confusion and added the following sentence into the manuscript “This was not due to inefficient function of the constructs as both DN constructs effectively reduced the kinase activity of their relevant Cdk (Suppl. Fig. S1).”

- Figure 7B was not intended to be a quantifiable analysis of Spy1 levels among breast cancer cell lines but rather to demonstrate that our functional data is carried out on cell lines expressing high levels of Spy1. This was added to the text “Spy1 levels have been demonstrated to be high in all proliferative normal mammary tissue [8], we ran a number of breast cancer lines together to select the lines expressing the highest level of Spy1 protein for functional analysis (Fig. 7B)”.

- Figures 6C and D represent triplicate transfections within experiments repeated twice. We have changed the figure to represent standard error within one representative experiment and stated in the text “Error bars reflect SE of triplicate transfections within one representative experiment. n=2”. These final 2 panels are supportive of the first 2 panels with regard to Spy1’s role on overriding FOXO function. Given that the data with all the other constructs closely resembles data has been published by Liu et. al. (Oncogene 2008, 27:4733-4744), and the statistical story for Spy1 overriding FOXO-induced apoptosis and the dependency on Cdk1 are addressed in the first 2 panels, representative experiments with ‘within’ experiment error are convincing.

3. The overall denso value between loading controls for different experiments reflected a large error, as one may expect. For this we presented one representative blot within Figure 2B and pointed this out in the figure legend “Western blot analysis of one representative lystate from A-B” This loading control follows the same trend that has been analyzed in detail in Figure 1.

4. Statistica is the program used ([http://www.statsoft.com/](http://www.statsoft.com/)). We changed the wording to “Statsoft’s STATISTICA” for clarification in the text.

Reviewer Partha Roy.

1. As suggested we re-measured our samples to reflect a volume measurement in mm$^3$. We have expressed this in Figure 3B in both overall measurements as well as a new panel showing the volume measurements over time. Area has been replaced for volume accordingly throughout in the text.

2. We have added in a discussion about the future relevance of conducting this in vivo using xenographs but felt this was beyond the scope of this specific story. At the end of the Results & Discussion: “Our results support the importance of further examining these effects in vivo. These future experiments are dependent on developing mechanisms of more homogeneous and stable Spy1 knockdown. “

3. As mentioned above and rationalized above, the loading control for Figure 2C was presented as a blot within the figure rather than its own panel with densitometry. We also used a blot from one of the repeat runs which more adequately reflects the overall densitometry values.
Thank-you again for this opportunity and for the time of the editors and reviewers as it has significantly improved our manuscript.

Sincerely,

Lisa A. Porter