Response to Reviewer.

1. The specific listing of controls and the detailed description of image analysis routines validate results from the screen and also inform the reader of the rigors in analysis. However, what was the rationale for incorporating such elaborate controls? What, if any, were the consequences of not including such controls (such as the details presented in the first paragraph on page 27)? How were the controls utilized to improve the confidence of detecting positive hits in the screen? Is it that if any of the above- mentioned controls did not produce the expected outcome, images acquired from the particular plate were not considered for further analysis?

   Amongst the many methods used to maintain quality control was the use of negative and positive controls. If the controls did not behave as expected, the entire plate was considered suspect and the set repeated. The consequences of not including the controls, in case by case basis is as follows:

   (i) dsRNA knockdown of VAP or GFP should lead to a dramatic reduction of aggregation. If this event did not happen, it suggested that RNA interference was not working in the plate. If 2 out of 3 replicates in a plate did not show lack of aggregation, data from the entire plate was not considered.

   (ii) Standard $CuSO_4$ controls (250 uM, 500 uM, 1000 um) were used to confirm the concentration dependence of VAP expression and resultant increase in aggregation. If the aggregation did not increase with increased CuSO4 then one would assume that either the cells were not responding to $Cu^{2+}$ or the CuSO4 stock had issues. The entire plate was discarded in this

scenario.

(iii) At the time of pilot screens in IISER, SOD1 and TDP43 were consistently showing increase in aggregation on knockdown of the respective genes. These genes were then kept as controls in each plate and were representative of repeats across plates; confirming that the data was reproducible over in terms of biological replicates and in a plate independent manner.

*We have included a line on the consequence of not including these controls on page 27.*

2. Results presented in the graphs should be better described to indicate if they were just means or means ± SD with specific mention of the number of trials. These need to be included in the legend for Figs. 3E, 4B, 4D, Fig. 9, Fig. 11B and 11D.

The error bars represent SD. Information has been added to all figure legends.

3. Perhaps a brief description of functions of Alsin and TBPH would go toward speculating the mechanisms by which these seemingly disparate gene products modify aggregation of VapB.

We have added background information on Alsin, SOD1 and TBPH in the discussion. A new figure (Figure 12), which attempts to link these loci together, has also been incorporated. The mechanistic reasons for change in aggregation state of $VAP^{P58S}$ on Alsin or TBPH knockdown is unclear at this point. For SOD1, our experiments suggest an

increase in superoxide levels cause a decrease in VAPP58S aggregation. This hypothesis is supported by studies using Paraquat, a ROS inducer.

5. Scale bar is missing in Fig. 3F.

   Correction made.

6. Fig. 3F provides confidence in the basis for the RNAi screen. Where are the aggregates present in the cells? Do they colocalize with any of the ER markers and do the modifiers affect the cellular distribution of VapB?

   Using immunofluorescence assays in S2R+ cells, I have seen that $VAP^{P58S}$-GFP aggregates co-localize with Calreticulin, an ER marker. These misfolded protein aggregates are also ubiquitinated and these assays were performed to characterize the generated cell line. We have not included these figures in the MS thesis as these experiments have are preliminary and have to be repeated with rigor.

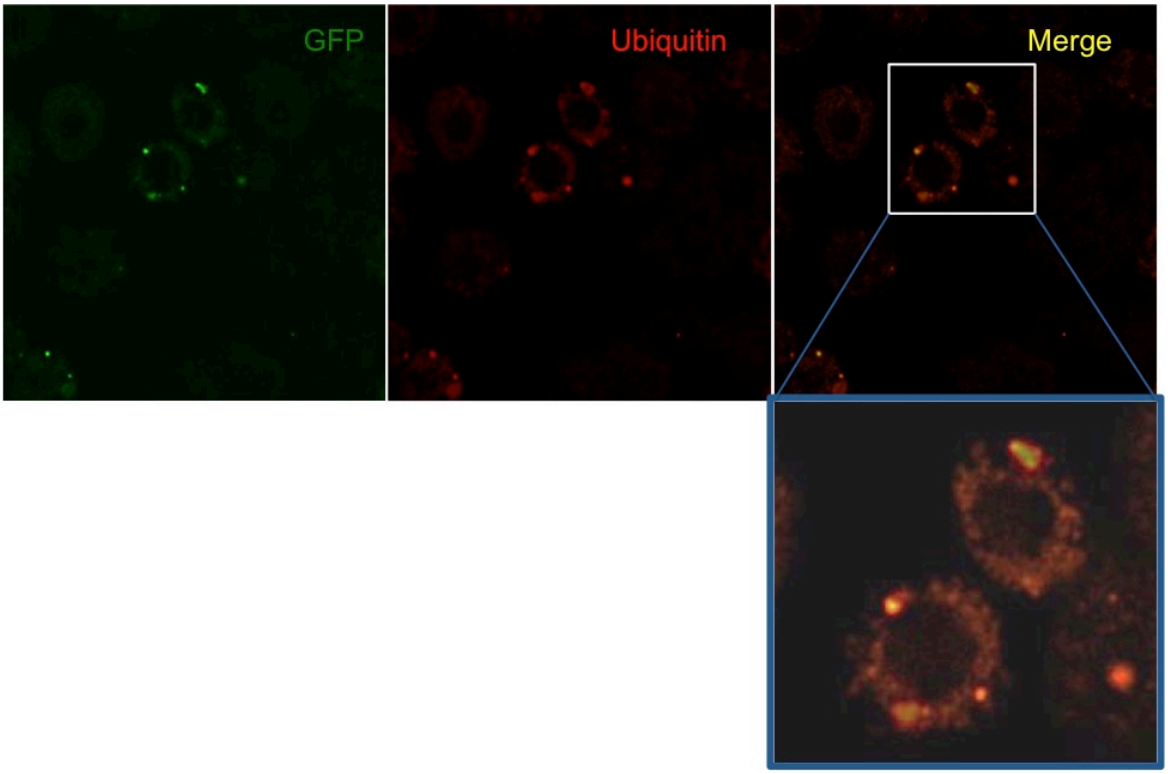


**Figure 1**: VAP$^{P58S}$GFP aggregates showed co-localization with ubiquitin with a co-localization co-efficient of 0.823 in S2R+ cells. Imaging was performed under confocal microscope at 63x. Mouse anti-ubiquitin (P4D1; SantaCruz) antibody was used to visualize ubiqtuitin (1:500 concentration). Images were analysed using JACoP plugin in ImageJ.