Response to Reviewers’ comments

Please note that our responses to the reviewers’ comments were shown in blue below. Our changes in the manuscript text were made using the “Track Changes” function.

Yue et al describes in this paper the consequences of IP3R deletion in ER calcium dynamics and homeostasis. The major finding is that there is an impaired ER Ca2+ leak and refilling in IP3R-TKO cells that might be linked to the increased expression of the ubiquitin ligase NEDD4L that promotes the degradation of Orai1 protein through proteasome. Unfortunately, the paper is not very interesting for major reasons:

1 All the observations are made in HEK293 cells genome-edited to deplete IP3Rs. Similar observations should be made in more relevant cell lines where depletion of IP3Rs have been revealed important (see any review in the references of the current manuscript).

We thank the reviewer for this great suggestion. If the main aim is to firmly establish the physiological rules of IP3Rs, then we totally agree with the reviewer that similar observations should also be made in more relevant cells, like DT40 cells, exocrine cells, and hippocampal neurons. However, the main purpose of the current manuscript is not the dissection of the physiological functions of IP3Rs, but to get a general understanding on the role of IP3Rs in ER-related Ca2+ signalling using a widely used mammalian model cellular system. Working with an easy-to-transfect, widely used cell line like HEK cells would thus serve us well for this purpose. We sincerely hope that the reviewer would agree with us on this point.

2 How many clones have been analyzed. In general CRISPR/Cas9 cell lines should be studied as single clones and several clones should be tested (at least for the most important observations) in order to confirm the results.

We thank the reviewer for pointing out this important issue, and we apologize for not presenting our data well enough. We actually used two separate stable cells, GCaMP and R-CEPIA1er cells, to do the genome editing. We then had at least two clones confirmed with sequencing for each type of cells (two GIPK clones and two RIPK clones). To reduce the workload, we only picked two different clones, one GIPK and one RIPK clone, for further testing. Assays like CCh-induced Ca2+ releases (Fig. 1A vs 1B), SOCE responses (Fig. 3A vs Fig S2A), proliferation and migration assays, and western analysis (Fig. S2C) were carried out in both clones. To make this point clear to readers, we have now added, “two different” into the first sentence of the result section: “……we made two different IP3R1-2-3 triple knockout HEK cell lines (IP3Rs-TKO)……” (Line 80); and “two separate” into the second sentence “These IP3Rs-TKO cells were generated from two separate HEK cell lines……” (Line 82).

3 The data about the role of NEDD4L in Ora1 degradation is probably the most interesting piece of data. However, the analysis of NEDD4L is very superficial. Control of NL.1 and NL.3 overexpression is not presented in the IBs and Loss of function as well as gain of function experiments should be performed to further link the expression of NEDD4L to Ora1 degradation.
We thank the reviewer for stating our “data about the role of NEDD4L in Orai1 degradation is probably the most interesting piece of data”.

We used fluorescence levels to indicate the expression level of mScarlet-P2A-NL.1 and mScarlet-P2A-NL.3. We have now followed the reviewer’s advice and included statistics showing fluorescence intensities of mScarlet to indicate the levels of NL.1 or NL.3 expression (left panel in Fig. 4C).

We also followed the reviewer’s advice and performed WESTERN analysis on NEDD4L expression in WT and GIPK cells. Unfortunately, the NEDD4L antibody we purchased did not work in our hands. As NL.1 or NL.3 only modulates the activities of NEDD4L, we did not perform analysis on the effects of NL.1 or NL.3 on the mRNA level of NEDD4L. Since we already showed that expression of IP$_3$R3 could alter mRNA levels of NEDD4L, and that modulating NEDD4L activities could change Orai1 expression and amplitude of SOCE responses, we feel that not showing NEDD4L protein levels won’t affect the integrity of this manuscript.

4- It is not clear how the depletion of IP3Rs can induce the transcription of NEDD4L. There are several transcription factors that are altered upon ER stress or IP3R deletion (i.e. NFAT). The authors should try to at least discuss a potential hypothesis. Better if they perform any experiment regarding this concern.

We have followed the reviewer’s advice and added the following sentences into the newly added discussion section: “The mechanistic underpinning of the linked expression between IP$_3$R3 and NEDD4L is yet to be established. It is likely that some IP$_3$R-dependent transcriptional factors might get involved and further investigations are needed to elucidate this” (Line 327-330). We didn’t perform NFAT translocation assay, as NFAT activity is also SOCE-dependent, and SOCE is reduced in IP$_3$Rs-TKO cells. This would make the interpretation of NFAT results difficult. We will pursue the underlying mechanisms in an ongoing project.