Reviewer 3

Introduction.

The introduction is a bit lengthy but at the same time misses sometimes detailed information to better inform the reader on the scientific background/rationale for the proposed hypothesis. E.g. line 44/45 – this statement could have been expanded on in more detail given that the authors hypothesis is based on this information.

As mentioned in the answer to the question of referee 1, we have rewritten the introduction in order to explain in more detail the scientific background and rationale for the proposed hypothesis

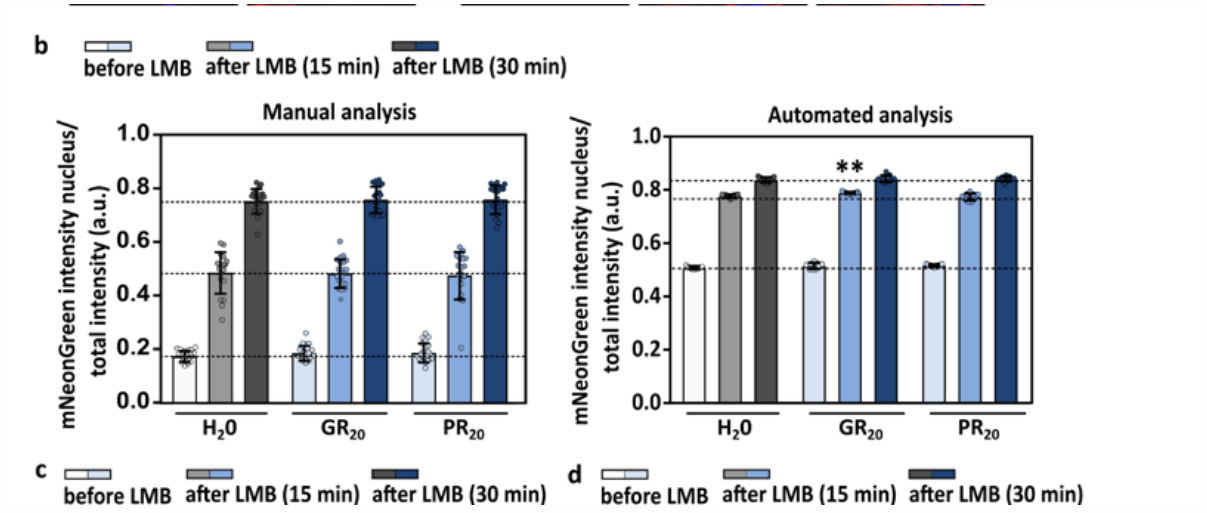
Methods.

Line 133-135 – how do the authors justify using the average of one microscopic image as one data point? Would one not consider using one cell as one data point?

What is suggested by the referee can indeed be done. However, the data points obtained from the individual cells can also not be considered in the statistical analysis as independent measurements. We decided to go for an intermediate solution avoiding as much as possible any selection bias by averaging each image as a data point, which might also not be perfect. This only applies to the limited number of manually analysed experiments shown in this manuscript and our way of analyzing the images is clearly documented in the M&M section. We decided to keep our initial analysis as it allows the comparison of our new data with previously published experiments performed in a similar way.

Line 136 – have the authors demonstrated that their high content imager generates equal data sets than their manual data analyses? If yes, this should probably be cited here.

We thank the referee for this remark. We have indeed compared the data obtained by manual analysis with those obtained by the high content imager and this was indeed be reported before in Vanneste et al., 2019 (ref 41 in the revised manuscript; Fig. 2b). We have added a sentence in the M&M section to clarify this: "As previously shown, the manual and automatic evaluation of nucleocytoplasmic transport resulted in similar results [41]."



Line 201 – the authors should describe how they quantify their western blot data. Further, an uncropped western blot should be provided in the supplement.

We have added some extra information in relation to the quantification of the Western blots ("Luminescent signals were analyzed using ImageQuant TL software version 7.0 (GE Healthcare) and

normalized to the loading control.). In addition, we have included the uncropped Western blots as supplementary Figure.

Results.

Line 222 - The reviewer is wondering if the authors actually know which specific importin alpha is responsible for the import of their reporter construct? This information could be helpful in fully understanding whether their assay only measures import through importin alpha 1 or whether other importins are involved in the measured import as presented.

We thank the referee for this important question. Importin alpha subtypes can be divided into 3 structural classes: class 1 containing importin alpha 5, 6 and 7; class 2 containing importin alpha 1 and 8 and class 3 containing importin alpha 3 and 4. Many cargos are not limited to one importin alpha or even structural class to be imported into the nucleus (Pumroy and Cingolani, Biochem. J; 2015). For example, in the case of SV40 NLS it has been shown that this NLS has in vitro affinity for importin alpha 1, 3, 4, 5 and 7 (Kelley et al. BMC Cell Biology 2010). However, there is a large variation in relative expression of different importin alpha subtypes between cell and tissue types and it is therefore very difficult to proof/predict the relative contribution of each different importin alpha subtype to the import of a specific cargo in a specific cell type. It is nonetheless safe to state that the reporter constructs in this paper are imported by several importin alpha subtypes, amongst which importin alpha 1.

Line 224 – did the authors actually look at TDP-43 co-localization to stress granules?

We didn't check the colocalization of TDP-43 to the stress granules in this study as this was already done before. There are indeed several published studies claiming that TDP-43 is indeed part of the stress granules (e.g. Colombrita, C., Zennaro, E., Fallini, C., et al. (2009). TDP-43 is recruited to stress granules in conditions of oxidative insult. J. Neurochem. 111, 1051–1061.). Moreover, we previously confirmed the fact that TDP-43 was localised to stress granules (Boenaems et al., 2017; ref 42).

Line 232 – figure 2B – it would be great to support this quantification with some images, maybe in the supplement (same for Figures 2 C+D).

These images are shown in Figure 2E.

Line 284 – this is in reference to a question from above – are the authors certain that only importin alpha 1 is involved in this transport – if not, could stress potentially switch the use of importin alpha subtypes?

As explained earlier, many cargos including the import reporters that we used can be imported by several importin alpha subtypes which could in theory take over the import function of importin alpha 1. However, in Hela cells, other importin alpha subtypes are generally expressed less than importin alpha 1. Moreover, from the Mammalian Stress Granule Database (MSGP; https://msgp.pt) it can be learned that several other importin alpha subtypes (importin alpha 4, 5 and 7) are also part of the stress granule proteome.

Line 295 – While the NLS-lacking GFP reporter shows reduced localization to stress granules, it still does co-localize with G3BP. Can the authors speculate on why this might be the case?

In our discussion we stated: "Moreover, it are exactly the NLS bearing stress granule proteins that mislocalize and aggregate in ALS which are substrates to this chaperone activity. This suggests that besides acting as a targeting sequence to the nucleus, NLSs may as well link aggregation-prone proteins to their transport receptors in cytoplasmic stress granules. In line with this, we observed that the stress granule localization of our reporter was dependent on the presence of its NLS.

The GFP reporter constructs localize in the stress granules through interaction of the NLS with receptors of the nucleocytoplasmic transport machinery such as importin beta or importin alpha. Other authors like Zhang et al., 2018 (Cell = ref 11) have also demonstrated the presence of Exportin-1 in these stress

granules. Interaction of the nuclear export signal (NES) of the GFP reporters used in our paper with their export receptor could therefore also explain the residual co-localization with G3BP in the GFP2x-NESikB2 construct.

Line 305 – this statement should be revisited and maybe toned down given that there is still reporter protein present in the nucleus suggesting that some nuclear import might still be occurring. The authors should acknowledge this and if needed discuss their conclusions in more detail.

We agree with the referee and have toned down or statement as still the majority of the reporter can get to the nucleus.

Line 317 and 353 – here, the authors should refer to supplemental figure 6 – without that figure, the reviewer immediately wondered if it would be helpful to stain for importins under the G3BP knockdown conditions, to determine if there is stress-induced aberrant nuclear pore protein machinery expression even in the absence of stress granules – which is exactly what the authors did in S6.

We agree that the information present in supplemental figure 6 could indeed be mentioned earlier in the result section. As a consequence, we have made a reference to this figure for the first time at the place indicated by the referee. As a consequence, we had to renumber the supplemental figures. The previous Fig. S6 became Fig. S4.

Line 396 – what is known about the stress granules that are formed due to overexpression of G3BP1? Are they similar to 'regular' stress granules?

Overexpression of G3BPs has been observed to drive stress-independent formation of stress granules that enrich for the classical SG factors (Matsuki et al., 2012 = ref 44; Reineke et al., 2012 Molecular Biology of the Cell; Yang et al., 2020 = ref 4 and many others). These findings are consistent with G3BP being a central and essential node in the SG interactome.

Line 401 – what happens to importin alphas?

We agree that this is a very good question. However, this quantification was not performed and it was unfortunately not possible to perform this experiment during the short period of time allocated to perform the revision of our manuscript.

Line 427 – the authors should reference figure 6D here

We apologize for not having mentioned this figure here. In addition, we have replaced KPNB1 by importin-β1.

Minor overall comments.

The font seems to change at varying locations throughout the manuscript.

The changes in the font were induced by formatting our manuscript into the format of the journal. We have carefully checked the entire manuscript and hope that we have corrected all these inconsistencies.

Supplemental figure 6 – image panels should be labeled with importin alpha 1 (not 2), correct? →

We apologize for this mistake and have corrected it to importin alpha 1.