The SAC1 domain in synaptojanin is required for autophagosome maturation at presynaptic terminals.

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1st Editorial Decision 20 October 2016

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find the analysis interesting and are in general supportive of publication here. They raise a number of constructive and specific issues that I anticipate that you will be able to sort out in a good manner. Given the referees' comments I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should point out that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to resolve the major concerns raised at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Let me know if we need to discuss anything further. Looking forward to seeing the revised version
REFEREE REPORTS

Referee #1:

In this well-written manuscript, the authors studied macroautophagy within presynaptic terminals and revealed a novel role for the Synaptojanin SAC1 domain and, in particular, the Parkinson's disease (PD)-causing mutation R258Q located in this domain. Although it was already reported recently that loss of Synaptojanin in zebrafish led to autophagy defects (George et al., 2016), the current study has provided additional mechanistic insights into Synaptojanin function and suggested a pathogenic mechanism for hereditary PD with Synj1 mutations. First, the authors performed a series of well-designed genetic experiments in Drosophila to show that the R258Q mutation does not affect synaptic vesicle endocytosis at the NMJ and photoreceptor neurons. Second, they showed that Synj1 is involved in presynaptic autophagy, consistent with George et al. (2016). Moreover, the normal function of the SAC1 domain is needed for autophagosome formation at presynaptic terminals. Third, the author provided evidence that Atg18a, a key PI(3)P-binding protein accumulated at synaptic boutons in R238Q mutants, consistent with the published notion that this mutation affected PI(3)P dephosphorylation. Fourth, the authors examined Atg18a-positive fine structures using TEM images and provided further evidence that the R238Q mutation altered an early step in autophagosome formation. Fifth, they provided some evidence to support the hypothesis that Synj1 regulates Atg18a uncoating at autophagosomal membranes. Finally, the authors showed that the R238Q mutation causes neurodegeneration and reduced animal survival under starvation conditions. Many of these experiments are well performed, and together, they have provided novel mechanistic insight into Synaptojanin function in autophagy and also highlight the role of presynaptic autophagy in PD pathogenesis.

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Referee #2:

In this report by Vanhauwaert and collaborators, a case is made for a role of the lipid phosphatase synaptojanin in synaptic macroautophagy and an impact of a Parkinson-linked mutation (R258Q) in the human ortholog (SYNJ1) on this process. Synaptojanin is best known for its actions on synaptic vesicle recycling, and Verstreken as well as several other investigators in the field have done seminal work in the past showing that a key role of synaptojanin at the synapse is to hydrolyze PI(4,5)P2 via its inositol 5-phosphatase domain, a step required for the efficient uncoating of clathrin-coated vesicles. Other studies have shown that the Sac1 domain of synaptojanin dephosphorylates a different set of phosphoinositides, including PI(3)P, a lipid controlling both the physiology of endosomes as well as the biogenesis and maturation of autophagosomes. While the nature of the substrates that are physiologically hydrolyzed by the Sac1 domain of synaptojanin is still unclear, there is good evidence that the yeast orthologs of synaptojanin can hydrolyze PI(3)P in cells. In fact, one study showed that hydrolysis of PI(3)P by yeast orthologs of synaptojanin and myotubularin is required to shed Atg proteins (including Atg18p) from autophagosomal membranes,
facilitating the completion of autophagy. Here the authors show that synaptic vesicle recycling is normal in flies expressing the synaptojanin R258Q mutant. However, they show that the same mutant accumulates Atg18 on nascent synaptic autophagosomes, blocking autophagosome maturation. They also find that these defects correlate with subtle neurodegeneration. This is generally an interesting manuscript that shows effects of a PD-linked mutation in synaptojanin that are distinct from those resulting from complete loss of synaptojanin or loss of the inositol 5-phosphatase activity, which is believed to target PI(4,5)P2. While there are several noteworthy observations showing a negative impact of the PD mutation on synaptic macroautophagy, additional work is required to make this study more compelling.

Specific concerns:
1. While starvation is an excellent trigger for autophagy, its (patho)physiological significance is unclear for neurons, particularly when the main goal of this study is to model Parkinson's. Therefore, to convincingly show that pathophysiological-relevant autophagy defects result from the expression of the synaptojanin R228Q mutant, the authors should examine paradigms of autophagy-mediated protein aggregate clearance, in addition to starvation-induced autophagy.

2. Given that the authors are attempting to clarify Parkinson disease mechanisms caused by the synaptojanin R228Q mutant, a validation in mammalian neurons is critical. Does expression of this mutant interfere with neuronal autophagy in a mammalian system?

3. There is no direct evidence that the defects in autophagy induced by the synaptojanin R228Q mutant expression cause any deleterious effects. The subtle fly viability/neurodegeneration phenotypes may have nothing to do with the subtle autophagy defects described by the authors. In order to connect these sets of observations, the authors should test whether enhancing autophagy can rescue the fly viability/neurodegeneration phenotypes.

4. Does the PD mutant R228Q behave like the Sac1-phosphatase dead mutant? If this is the assumption made by the authors, they should demonstrate it in their most robust functional assays, rather than extrapolating from the (thin) published literature on the topic.

5. Since the Sac1 domain of synaptojanin can dephosphorylate several substrates, two of which can bind to Atg18 (PI(3)P and PI(3,5)P2), it is unclear whether the relevant substrate accumulating in the synaptojanin R228Q mutant is PI(3)P or PI(3,5)P2. Can the authors determine which one is relevant using Vps34 or Pikfyve blockers (or equivalent genetic manipulations)? There is now good evidence from the literature showing that excess PI(3)P resulting from impaired dephosphorylation can be counteracted by decreased synthesis.

Other issues:
6. The immunoisolation experiment in Figure 2B has no negative control. To show the specificity of the synaptojanin 1 binding to Atg9-positive organelles, it is important to show that other synaptic proteins are not co-immunoisolated.

7. In Figure 2M-O, the chloroquine experiment should be done also under starvation conditions, where the largest increases in Atg8 fluorescence are expected to occur.

Referee #3:

Synaptojanin is a phosphoinositide phosphatase that has been shown to play an important role in synaptic vesicle recycling. Synj contains two enzymatic PIP phosphatase domains, the 5' phosphatase domain and the SAC1 domain. It is well characterized that 5' phosphatase activity regulates clathrin-mediated endocytosis via dephosphorylating PI(4,5)P2. However, the role of SAC1 domain in Synj remains largely unknown. Autophagy is a highly conserved intracellular degradation process and has been implicated in various neurodegenerative diseases. Here the authors found that Synj is required for autophagosome formation in presynaptic terminals, and this function is inhibited by the Parkinson's disease Synj-R258Q mutation. R258Q affects the SAC1 domain function and caused accumulation of the PI(3)P-binding protein Atg18a on nascent synaptic autophagosomes. They further showed that Synj-RQ mutation caused shortened lifespan and dopaminergic neuron degeneration in fly brain. Overall, the findings are interesting and the authors
present the results in a clear and concise manner. Addressing the following issues would further strengthen this manuscript.

Major comments:
1. In this study, the function of SAC1 domain was determined based only on the R258Q mutation. However, the biochemical nature of this mutation remains unclear. It has been shown that mutating the conserved catalytic cysteine of Synj-SAC1 domain results in a catalytically dead SAC1 domain in mouse and human Synj. In addition to R258Q mutation, the authors should also assess the role of SAC1 domain in Synj using the catalytic dead mutation.

2. Authors showed that Synj interacted with Atg9 and colocalized in synaptic boutons. Does Synj-RQ also interact with Atg9? It has been shown that Atg2-Atg18 complex regulates Atg9 recycling during autophagy. Does Synj-Atg9 interaction contributes to Atg18 function in synapse?

3. The authors should use GFP-mcherry-Atg8 for flux assay in Fig 2M-O.

4. Besides GFP-Atg18a, the authors should show the accumulation of PI3P in Synj-RQ mutant.

5. It remains unclear on how Synj-RQ affected autophagosome formation. Can Atg18 knockdown or reduce the level of PI3P suppress Synj-RQ induced neurodegeneration in Fig5?

Referee #1:

In this well-written manuscript, the authors studied macroautophagy within presynaptic terminals and revealed a novel role for the Synaptotagmin SAC1 domain and, in particular, the Parkinson's disease (PD)-causing mutation R258Q located in this domain. Although it was already reported recently that loss of Synaptotagmin in zebrafish led to autophagy defects (George et al., 2016), the current study has provided additional mechanistic insights into Synaptotagmin function and suggested a pathogenic mechanism for hereditary PD with Synj1 mutations. First, the authors performed a series of well-designed genetic experiments in Drosophila to show that the R258Q mutation does not affect synaptic vesicle endocytosis at the NMJ and photoreceptor neurons. Second, they showed that Synj1 is involved in presynaptic autophagy, consistent with George et al. (2016). Moreover, the normal function of the SAC1 domain is needed for autophagosome formation at presynaptic terminals. Third, the author provided evidence that Atg18a, a key PI(3)P-binding protein accumulated at synaptic boutons in R238Q mutants, consistent with the published notion that this mutation affected PI(3)P dephosphorylation. Fourth, the authors examined Atg18a-positive fine structures using TEM images and provided further evidence that the R238Q mutation altered an early step in autophagosome formation. Fifth, they provided some evidence to support the hypothesis that Synj1 regulates Atg18a uncoating at autophagosomal membranes. Finally, the authors showed that the R238Q mutation causes neurodegeneration and reduced animal survival under starvation conditions. Many of these experiments are well performed, and together, they have provided novel mechanistic insight into Synaptotagmin function in autophagy and also highlight the role of presynaptic autophagy in PD pathogenesis.

Other comments:

1. The experiments to examine the interaction between Synj and Atg9 are not quite convincing. Figure 2A is not very useful. Both proteins are broadly distributed within presynaptic boutons, so it is not useful to support the notion that these two proteins interact. Atg9 was ectopically overexpressed. Could the authors examine endogenous Atg9? This point is more important for the co-IP experiment. A better western blot gel image should be provided. It is unclear what the lane between "Flow" and "IP" is. Also some Synj signal is visible in it. To address the reviewer’s concern we tried several anti-Atg9 antibodies but none are specific. We then resorted to a genomic Atg9HA construct where Atg9HA is expressed under endogenous promoter control. The distribution of Atg9HA is similarly broad (Figure R1 and Figure S2A-A”), consistent with the idea that Atg9 decorates early autophagic vesicles and autophagosomes. We then used this Atg9HA to repeat the co-IP experiments. We used anti-HA to IP neuronal autophagosomal...
membranes (Atg9 positive) from fly heads expressing Atg9HA. We find that Synj co-IPs with this fraction (Figure R1 and Figure 2A). As additional control, we also included other synaptic proteins (alpha-SNAP and Complexin) that do not co-IP with Atg9HA. (Figure R1 and Figure 2A). These data replace the data in the original manuscript and we moved the immunolabeling of Atg9HA with anti-HA antibodies to the supplemental section.

Figure R1, as adapted on Figure 2 and S2. Synaptojanin localizes to Atg9 positive membranes.

(A-A”) Synaptic NMJ boutons expressing under endogenous promotor control the preautophagosomal marker Atg9HA (Atg9-;Atg9HA) and immunolabelled with anti-Synj and anti-HA antibodies, scale bar 5 µm. (B) Western blot of an anti-HA immunoprecipitation from control fly heads (w1118), Atg9HA expressing fly heads (w;;Atg9HA) and w;synjRQ;Atg9HA. Blots probed with anti-Synj to assess if Synj (predicted 134kDa) is present on Atg9 positive structures; anti-HA to assess immunoprecipitation specificity, Atg9HA (predicted 96 kDa). As control alpha-SNAP (33kDa) and Complexin (18kDa) were probed. Experiments were performed in independent duplicates.

2. Figure 2G, L and O can be confusing to some readers. It is better to present grey and black columns separately instead of stacking them together.
We have separated Fed and Starved in Figure 2G, M; Figure S2F and Figure 3E.

3. On Page 8, the authors should note that their conclusion that Synj is involved in autophagy is consistent with the recent report by George et al. 2016.
We have added this reference and adapted the text.

4. Figure 2N is not very convincing. A number of Atg8 dots (although with less intensity) are visible, but they are not labeled with arrowheads.
We adapted the image using the LUT of 16-colors to clarify that in synjRQ boutons Atg8 does not obviously relocalize into dots as is the case in the controls (Figure 2B-F’). To avoid being biased when quantifying the data, the genotypes were blinded, and the dots we included in the quantification were defined by the particle analysis tool of a binary image. Threshold settings were identical for all images included in the quantification. Compared to controls after starvation or stimulation, this analysis method did not show an increase in the number of Atg8 dots in fed or starved synjRQ with or without treatment with chloroquine (also fed or starved) (Figure 2K-M).

5. It is unclear why Figure 2M and N are not colored as those for Lamp1 and Atg18.
As indicated above, we adapted the Figure using our LUT; please see Figure 2B-F’ and K-L.

6. The evidence suggesting that Synj regulates Atg18a uncoating at autophagosomal membranes seems to be limited. Since this is one of the key mechanistic studies, it would be nice if the authors could perform additional experiments to strengthen this point.
To address this point, we constructed new transgenic flies that allow us to express mEos3.2-Atg18a under the control of Gal4. mEos is a photoconvertible fluorescent protein that shifts from green to red fluorescence when activated.

In a first set of experiments we used the mEos3.2-Atg18a flies and converted only single molecules of mEos3.2-Atg18a at the NMJ, allowing us to track individual proteins in time. This enabled us to assess if Atg18a mobility is altered upon induction of starvation and if Atg18a is recruited to
autophagosomes. We find that Atg18a is less mobile after autophagy induction of autophagy, consistent with Atg18a binding to autophagosome membranes (Figure R2 and Figure 4F-J).

Having established this, we then expressed mEos3.2-Atg18a in control and synjRQ knock in flies. We converted a boutonic pool of mEos3.2-Atg18a, and assessed the rate at which Atg18a positive organelles in the neighbouring boutons acquire red fluorescence. The acquisition of red fluorescence in neighbouring boutons is dependent on (1) diffusion of red Atg18a from the site of conversion to the neighbouring boutons; and (2) the exchange rate of Atg18a on nascent autophagosomes. Given that NMJ morphology in controls and SynjRQ mutants is very similar, we do not expect diffusion of Atg18a between boutons to be majorly affected. Hence, if the exchange of Atg18a at nascent autophagosomes is impeded in synjRQ mutants we expect them to acquire red fluorescence slower than in controls.

We conducted this experiment and in line with the model outlined above we indeed see that photoconverted red Atg18a accumulates less quickly on Atg18a positive organelles in SynjRQ compared to controls (Figure R2 and Figure 4K-M). We also quantified the amount of cytoplasmic red Atg18a in boutons neighbouring the site of photoconversion and found this to be equal in controls and SynjRQ suggesting that diffusion between boutons per se, is indeed not affected (not shown).

We believe these data provide further evidence that Atg18a exchange at the membrane of nascent autophagosomes is regulated by Synaptotagmin, consistent with the model that phosphoinositide conversion regulated by Synaptotagmin also regulates protein-coating of autophagosomal membranes, similar to its role during endocytosis.

Figure R2, added to Figure 4. Synaptotagmin regulates Atg18a uncoating at autophagosomal membranes.
(A-E) Transgenic larvae expressing Atg18amEos3.2 were imaged at 33 Hz. (A-A’’) Representative low-resolution image of NMJ bouton (A), sptPalm trajectory map (A’), average intensity (A’’) and diffusion coefficient (A’’’) of Atg18amEos3.2. Scale bar 5µm. (B) Comparison of MSD of Atg18amEos3.2 in fed and starved conditions (n≥6 larvae and n≥18 NMJ per condition) (C) Analysis of the area under the MSD curve (µm2s) showed significant decrease in starved compared to fed controls. (D) Diffusion coefficient distribution of Atg18amEos3.2 in fed and starved controls. (E) Mobile to immobile ratio for fed (13.4±1.8) significantly reduced in starved conditions (7.1±0.5). Statistical analysis by one-way ANOVA with Tukey’s multiple comparison test **P<0.01. Mean and standard error of mean are plotted. (F-G) Representative time lapse images of mEOS3.2 photoconversion experiment were the RFP fluorescence recovery on an individual Atg18amEOS3.2 after GFP conversion of the neighboring bouton. An individual Atg18amEOS3.2 dot (D42-Gal4- Atg18amEOS3.2) was monitored at a synaptic bouton of control (F) and synjRQ (G). (H) Quantification of the ratio RFP/GFP fluorescence intensities of an Atg18amEOS3.2 dot in the indicated genotypes (in % of final time point (10min after photoconversion) RFP/GFP fluorescence ratio of the Atg18amEOS3.2 dot), error bars represent SEM, scale bar 2µm. Full genotypes appear in the supplement. Statistical analysis comparing curves fitted by linear regression. n≥3 larvae and n≥13 NMJs per genotype. Note the reduced RFP/GFP fluorescence recovery of Atg18amEOS3.2 in synjRQ compared to wild-type controls.

Referee #2:

In this report by Vanhauwaert and collaborators, a case is made for a role of the lipid phosphatase synaptojanin in synaptic macroautophagy and an impact of a Parkinson-linked mutation (R258Q) in the human ortholog (SYNJ1) on this process. Synaptojanin is best known for its actions on synaptic vesicle recycling, and Verstreken as well as several other investigators in the field have done seminal work in the past showing that a key role of synaptojanin at the synapse is to hydrolyze PI(4,5)P2 via its inositol 5-phosphatase domain, a step required for the efficient uncoating of clathrin-coated vesicles. Other studies have shown that the Sac1 domain of synaptojanin dephosphorylates a different set of phosphoinositides, including PI(3)P, a lipid controlling both the physiology of endosomes as well as the biogenesis and maturation of autophagosomes. While the nature of the substrates that are physiologically hydrolyzed by the Sac1 domain of synaptojanin is still unclear, there is good evidence that the yeast orthologs of synaptojanin and myotubularin is required to shed Atg proteins (including Atg18p) from autophagosomal membranes, facilitating the completion of autophagy. Here the authors show that synaptic vesicle recycling is normal in flies expressing the synaptojanin R258Q mutant. However, they show that the same mutant accumulates Atg18 on nascent synaptic autophagosomes, blocking autophagosome maturation. They also find that these defects correlate with subtle neurodegeneration. This is generally an interesting manuscript that shows effects of a PD-linked mutation in synaptojanin that are distinct from those resulting from complete loss of synaptojanin or loss of the inositol 5-phosphatase activity, which is believed to target PI(4,5)P2. While there are several noteworthy observations showing a negative impact of the PD mutation on synaptic macroautophagy, additional work is required to make this study more compelling.

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We have been asking ourselves the same question when preparing this work, but realized that it comes with a number of very uncertain assumptions. In discussing with neuropathologists, it is clear that not all cases of Parkinson’s show overt accumulation of protein aggregation (ie Lewy bodies). Specifically for the case of SYNJ1R258Q mutation carriers, it is unknown if these patients suffer from synuclein-positive (Lewy bodies) or tau-positive (neurofibrillary tangles) aggregates. Future autopsies will be needed to reveal the pathology.
There is a single child with a homozygous truncating mutation in SYNJ1 that caused a severe loss of function, who did come to autopsy. Here, tau-positive pathology was described in his brain. However, it is important to realize that this child carried non-Parkinson causing mutations in SYNJ1 and instead suffered from severe epileptic encephalopathy because of severe loss of SYNJ1 protein function (Dyment et al. 2015).

Nonetheless, to start addressing the point raised by the reviewer we conducted to additional experiments:

First, we assessed autophagy-mediated protein clearance at the level of p62 positive aggregates. We compared p62 levels in the insoluble fraction on western blot of 30 day old and 3 day young synjRQ and control (w1118) fly heads. However, p62 levels in the insoluble fraction increased during aging to a similar extend in both control and synjRQ mutants (Figure R3). We conclude that Synaptojanin does not affect the clearance of p62 positive aggregates in fly brains. There can be multiple reasons for this: for example the autophagic pathway controlled by synaptojanin is different than the one used for p62 clearance; alternatively, the differences are below our detection limit. More work will be needed to sort out if synaptojanin-dependent autophagy is involved in aggregate clearance and if so, what aggregates are being cleared by this pathway.

Second, in line with the reviewer’s question, we also used a ‘more physiological’ means to induce autophagy and resorted to electrical motor neuron stimulation (30min 20Hz) in both synjRQ and controls. As we described recently, this paradigm induces synaptic autophagy in boutons (Soukup et al. 2016). We observed a clear increase in the number of Atg8mCherry dots in controls. In contrast, we do not observe such an increase in synjRQ (Figure R3 and Figure 2H-J).

Figure R3, C-E added to Figure 2. Paradigms of autophagy-mediated protein aggregate clearance and electrical stimulation induced autophagy.

(A-B) Western blot for p62 (+/- 100 kDa) using anti-p62 antibodies and for Tubulin (50 kDa) using anti-Tubulin antibodies as a loading control of the indicated genotypes (A). Quantification of Synj expression levels of the indicated genotypes (B). n≥ 10 fly heads per genotype. Experiments were performed in independent duplicates. Error bars represent the SEM. (C-E) Live Atg8mCherry imaging following 30 min of 20 Hz electrical nerve stimulation of indicated genotypes (C,D). Full genotypes are included in the supplemental section. Quantification of Atg8mCherry dots (arrows) (E). Statistical analysis by t-test **=p<0.01, n≥8 larvae and n≥33 NMJs per genotype. Error bars represent the SEM, scale bar 5 µm.

2. Given that the authors are attempting to clarify Parkinson disease mechanisms caused by the synaptojanin R228Q mutant, a validation in mammalian neurons is critical. Does expression of this mutant interfere with neuronal autophagy in a mammalian system?

To address this question we resorted to induced pluripotent stem cells obtained from two different patients carrying the R258Q mutation in SYNJ1 as well as two age-matched controls. These iPSCs
were differentiated into neurons using established methodologies ((Warlich et al. 2011; de Esch et al. 2014); materials and methods). Following validation of the identity of these cells (Figure R4, R5 and Figure 6, S4) these neurons were labelled with anti-WIPI2 (mammalian Atg18a) and the number of WIPI2 dots in neurites was counted under both in fed and starved conditions (quantification was done with blinding for genotypes). In line with our observations made with the fly model, we find a significant increase in the number of WIPI2 dots in fed SYNJ1RQ neurons compared to controls (Figure R5 and Figure 6). These data are in further support that in cells with PD-mutant SYNJ1 Atg18a/WIPI2 accumulates on nascent autophagosomes.

Figure R4, additional Figure S4. Characterisation of undifferentiated iPSC and embryoid bodies from patients carrying the SYNJ1 R258Q mutation and controls.

(A) The Italian pedigree with the SYNJ1 mutation. The black symbols denote individuals affected by juvenile parkinsonism. (B) Electropherogram showing the homozygous SYNJ1 c.773G>A (p.Arg258Gln) mutation present in the patient iPSC lines. (C-J) Immunocytochemistry analysis of patient iPSCs, showing expression of pluripotency markers. C-F are stained with anti-Nanog (red) and counterstained with DAPI (blue) and represent lines NAPO16.3, NAPO16.10, NAPO17.4 and NAPO17.5. Images G-J are stained with anti-OCT4 (green) and anti-Tra1-81 (red) and represent lines NAPO16.3, NAPO16.10, NAPO17.4 and NAPO17.5. Scale bars, 100 µm. (K) Expression analysis by RT-Q-PCR of pluripotency markers in the patient and control iPSCs. (L) Expression
analysis by RT-Q-PCR of differentiation markers in 8 day old Embryoid bodies from the patient and control iPSCs.

**Figure R5, additional Figure 6.** Human SYNJ1 regulates WIPI2/Atg18a in induced neurons derived from patients carrying the SYNJ1 R258Q mutation.

(A-B) Immunocytochemistry analysis of induced neurons (after 28 days of differentiation) showing expression of neuronal markers, anti-MAP2 (magenta) and anti-Synapsin1 (green) in control (A-A') and SYNJ1RQ (B-B'). Scale bars, 15 µm. (C-G) Images of induced neurons stained with anti-WIPI2 in fed and starved condition of controls (C,D) and SYNJ1R258Q (E,F). (G) Quantification of the number of WIPI2 dots of indicated genotypes (2 control lines and 2 patients lines (NAPO16 and 17), circle and triangle indicate separate lines for each genotype) under fed and starved conditions. Statistical analysis with 1-way ANOVA Kruskal-Wallis followed by Post Hoc test Dunn's multiple comparisons, ****=p<0.0001, **=p<0.01, ns, not significant. n≥21 images per condition per genotype. Error bars represent the SEM, scale bar 15 µm. Note that SYNJ1RQ has increased number of WIPI2 dots under fed conditions.

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To address this point we have performed a genetic interaction experiment: Atg18a accumulates in synjRQ mutants and we reasoned that genetically removing one copy of atg18a could rescue (partly) the observed phenotypes at the level of starvation induced survival (complete removal of atg18a is lethal).

We find that synjRQ; atg18a/+ animals on complete starvation live significantly longer than synjRQ animals, in line with the expectation. The problem here is that atg18a/+ animals alone live longer than the controls on complete starvation, making the results complicated to interpret. We also
wanted to address neurodegeneration of dopaminergic neurons, but the heterozygous *atg18a* animals have a significantly lower number of dopaminergic neurons, making also the interpretation of this phenotype in this genetic background difficult (data not shown). We hope the reviewer agrees it is better at this stage to not include these data in the main manuscript but we do present the starvation data here to the reviewers (Figure R6).

We did not try to enhance autophagy genetically as suggested because we’d need to identify a means to do so downstream of the role of Atg18a and were not sure what manipulation to use.

**Figure R6. Genetically removing one copy of atg18a could rescue (partly) the complete starvation phenotype.**

(A) Complete starvation (water only diet) assay in *synjRQ* knock-in animals (light green), Controls (*w1118*, black), *synjRQ* that lost one copy of Atg18a (dark green) and *Atg18*+/+. All survival curves are **** significantly different from each other. Statistical analysis by comparing survival curves to each other by Log-rank (Mantel-Cox) test, ****=p<0.0001, n ≥ 62 flies per genotype, dotted lines surrounding the curves indicate the SEM.

4. Does the PD mutant R228Q behave like the Sac1-phosphatase dead mutant? If this is the assumption made by the authors, they should demonstrate it in their most robust functional assays, rather than extrapolating from the (thin) published literature on the topic.

We agree with the reviewer and have generated flies that express the *Drosophila* SAC1 dead mutant SynjC396S (SynjCS) under UAS control. We expressed SynjCS in *synj*-/− null mutants under the same conditions as used to express SynjRQ (D42-Gal4). We then assessed Atg8mCherry localization in *synj* mutants that express SynjCS and found that similar to our observations in *synj* mutants that express SynjRQ, Atg8 also does not concentrate in dots upon induction of starvation (Figure R7 and Figure 2). These data are in further support of the notion that Synj SAC1 domain function is required for synaptic autophagy and that the pathogenic mutant in Synj behaves in this respect similar to a Synj-SAC1 domain loss of function mutant.

**Figure R7, added to Figure 2. Synaptojanin SAC1 domain is required for synaptic autophagy.**
(A–E′) Live imaging of fed (A,B,C,D,E) and 4 h-starved (A′,B′,C′,D′,E′) NMJ boutons of Control (D42-Gal4>Atg8mCherry) (A–A′) and synj−/− null mutants (B,B′) either expressing synjRQ (C,C′), synjCS (D–D′) or synj+ (E,E′) and also expressing Atg8mCherry (D42-Gal4>Atg8mCherry). Full genotypes are included in the supplemental section. Quantification of the number of Atg8mCherry dots (arrows) (F). Statistical analysis with 1-way ANOVA Kruskal-Wallis followed by Post Hoc test Dunn’s multiple comparisons, ****=p<0.0001, ***=p<0.001, and each genotype individually fed and starved by t-test, ****=p<0.0001, n≥9 larvae and n≥18 NMJs per genotype. Error bars represent SEM, scale bar 5 µm. Note that loss of Synj blocks the formation of Atg8mCherry dots and this is not rescued by expression of synjRQ.

5. Since the Sac1 domain of synaptojanin can dephosphorylate several substrates, two of which can bind to Atg18 (PI(3)P and PI(3,5)P2), it is unclear whether the relevant substrate accumulating in the synaptojanin R228Q mutant is PI(3)P or PI(3,5)P2. Can the authors determine which one is relevant using Vps34 or Pikfyve blockers (or equivalent genetic manipulations)? There is now good evidence from the literature showing that excess PI(3)P resulting from impaired dephosphorylation can be counteracted by decreased synthesis.

We thank the reviewer for this comment. The Synj SAC1 domain is indeed capable of dephosphorylating several substrates, including PI(3)P and PI(3,5)P2, both of which can bind with Atg18a (Krick et al. 2006; Obara et al. 2008; Watanabe et al. 2012; Baskaran et al. 2012). To assess if PI(3)P and/or PI(3,5)P2 accumulate in synjRQ mutants we used pharmacology to block the production of either PI(3)P or PI(3,5)P2 in synjRQ mutants and monitored the accumulation of Atg18aGFP.

We used two drugs that are known to block kinases that produce PI(3)P (Vps34, blocked by wortmannin – WM) or PI(3,5)P2 (PIKfyve, blocked by YM201636 - YM). We first applied these drugs during starvation to control animals that express Atg18aGFP. While Atg18aGFP accumulates in dots in controls that are starved, application of either drug strongly blocks this effect and Atg18aGFP does not accumulate anymore, suggesting that Atg18aGFP can likely bind both PI(3)P and PI(3,5)P2 during autophagy at synapses (Figure R8 and Figure S3).

In synjRQ mutants the number of Atg18aGFP dots is always upregulated. However, when we starve the larvae in the presence of either WM or YM, the number of Atg18aGFP is lower than in untreated starved synjRQ mutants. These data suggest that, consistent with the previously established function of the Synj SAC1 domain, both PI(3)P and PI(3,5)P2 accumulate and are substrates for Atg18a in synjRQ mutants (Figure R8 and S3).

**Figure R8, added to Figure S3, PI(3)P and PI(3,5)P2 accumulate and are substrates for Atg18a in synjRQ mutants.**
Live imaging NMJ boutons in Controls (D42-Gal4>Atg18aGFP) and synjRQ also expressing Atg18aGFP. (A) Quantification of the number of Atg18aGFP dots of indicated genotypes under either fed, starved or starvation conditions combined with drugs that either block kinases that produce PI(3)P, wortmannin (WM) or PI(3,5)P2, YM201636 (YM) (data of Fed and Starved Control obtained previously Figure 3E). Statistical analysis by t-test comparing each genotype individually, **p<0.01 and *p<0.05, n≥7 larvae and n≥27 NMJs per genotype. Error bars represent the SEM. Note that both PI(3)P and PI(3,5)P2 accumulate and are substrates for Atg18a both in controls and synjRQ mutants.

Other issues:
6. The immunoisolation experiment in Figure 2B has no negative control. To show the specificity of the synaptojanin 1 binding to Atg9-positive organelles, it is important to show that other synaptic proteins are not co-immunoisolated.
We have repeated the IP with flies expressing endogenous Atg9HA. We used anti-HA to immunoprecipitate neuronal autophagosomal membranes (Atg9 positive) from fly heads expressing Atg9HA. Also under these endogenous conditions we detect the co-immunoprecipitation of Synj in the Atg9-positive membrane fraction (Figure R1 and Figure 2A). As requested we also tested if these immunoprecipitates are positive for other synaptic proteins (alpha-SNAP and Complexin) but did not detect these proteins (Figure R1 and Figure 2A). We also included a negative control: We repeated the IP with w1118 fly heads and probed for HA and Synj, whereas no HA was detected also no Synj was detected in w1118 immunoprecipitates. Together these data indicate specificity: We now show that Atg9 immunoprecipitates are not positive for the synaptic proteins alpha-SNAP and Complexin and that Synj does not co-immunoprecipitate in the negative control (Figure R1 and Figure 2A).

7. In Figure 2M-O, the chloroquine experiment should be done also under starvation conditions, where the largest increases in Atg8 fluorescence are expected to occur.
We have repeated the chloroquine (CQ) experiment but now under starvation conditions. As indicated in Figure R9 (and Figure 2K-M), CQ in controls under starved conditions results in an even larger increase in the number of Atg8mCherry dots compared to application of CQ under fed conditions. In contrast, the number of Atg8mCherry dots in starved synjRQ animals treated with CQ is still not significantly increased compared to untreated synjRQ mutants (Figure R8). These data further support the conclusion that autophagic flux is blocked in synjRQ.

Figure R9, added to Figure 2. Chloroquine experiment under starvation conditions.
(A-C) Images of NMJ boutons of synj−/− null mutants either expressing synj+ (A) or synjRQ (B) and also expressing Atg8mCherry (D42-Gal4>Atg8mCherry) fed or starved for 4h with chloroquine and labelled with anti-mCherry antibodies. Quantification of the number of Atg8mCherry dots (arrows) of indicated genotypes (in detail in the supplemental section) (C). Statistical analysis with 1-way ANOVA Kruskal-Wallis followed by Post Hoc test Dunn's multiple comparisons, ****=p<0.0001, **=p<0.01, n≥9 larvae and n≥18 NMJs per genotype. Error bars represent the SEM, scale bar 5 µm. Despite that chloroquine blocks autophagosome to lysosome fusion, Atg8mCherry dots still do not form in animals expressing synjRQ.

Referee #3:

Synaptotagmin is a phosphoinositide phosphatase that has been shown to play an important role in synaptic vesicle recycling. Synj contains two enzymatic PIP phosphatase domains, the 5' phosphatase domain and the SAC1 domain. It is well characterized that 5' phosphatase activity regulates clathrin-mediated endocytosis via dephosphorylating PI(4,5)P2. However, the role of SAC1 domain in Synj remains largely unknown. Autophagy is a highly conserved intracellular degradation process and has been implicated in various neurodegenerative diseases. Here the authors found that Synj is required for autophagosome formation in presynaptic terminals, and this function is inhibited by the Parkinson's disease Synj-R258Q mutation. R258Q affects the SAC1 domain function and caused accumulation of the PI(3)P-binding protein Atg18a on nascent synaptic autophagosomes. They further showed that Synj-RQ mutation caused shortened lifespan and dopaminergic neuron degeneration in fly brain. Overall, the findings are interesting and the authors present the results in a clear and concise manner. Addressing the following issues would further strengthen this manuscript.

Major comments:

1. In this study, the function of SAC1 domain was determined based only on the R258Q mutation. However, the biochemical nature of this mutation remains unclear. It has been shown that mutating the conserved catalytic cysteine of Synj-SAC1 domain results in a catalytically dead SAC1 domain in mouse and human Synj. In addition to R258Q mutation, the authors should also assess the role of SAC1 domain in Synj using the catalytic dead mutation. This is an excellent suggestion also raised by reviewer 2. We have generated flies that express the SAC1 dead mutant SynjC396S (SynjCS) under UAS control. We expressed SynjCS in synj−/− null mutants under the same conditions as used to express SynjRQ using D42-Gal4. Similar to SynjRQ, SynjCS localizes to synaptic boutons indicating the mutant protein is trafficked to synapses. We then assessed Atg8mCherry localization in synj mutants that express SynjCS and found that similar to our observations in synj mutants that express SynjRQ, Atg8 also does not concentrate in dots upon induction of starvation (Figure R7 and Figure 2E,E',G). These data are in further support of the notion that Synj SAC1 domain function is required for synaptic autophagy and that the pathogenic mutant in Synj behaves in this respect similar to a Synj-SAC1 domain mutant.

2. Authors showed that Synj interacted with Atg9 and colocalized in synaptic boutons. Does Synj-RQ also interact with Atg9? It has been shown that Atg2-Atg18 complex regulates Atg9 recycling during autophagy. Does Synj-Atg9 interaction contributes to Atg18 function in synapse?

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We repeated the immunoprecipitation experiment with synjRQ flies expressing endogenous Atg9HA. We used anti-HA to immunoprecipitate neuronal autophagosomal membranes (Atg9 positive) from fly heads expressing Atg9HA. Similar to Atg9 immunoprecipitations from control animals we detect Synj in Atg9 immunoprecipitates from synjRQ mutants (Figure R1 and Figure 2A). As a control indicating specificity, other synaptic proteins (alpha-SNAP and Complexin) did not co-immunoprecipitate (Figure R1 and Figure 2A). Hence, also the mutant SynjRQ protein resides on Atg9 positive membranes. We would like also clarify one issue: the conditions we used for immunoprecipitation indicate that Synj associates with Atg9 positive membranes but they do not allow us to conclude on direct binding of Synj to Atg9.

3. The authors should use GFP-mcherry-Atg8 for flux assay in Fig 2M-O.
To assess this question, we generated flies that express GFP-mCherry-Atg8 under UAS control and subsequently crossed these in the synjRQ background. After starvation we quantified the number of yellow Atg8 dots (where GFP is not yet quenched) and the number of red dots (where GFP is quenched and only RFP fluorescence remains). In controls we find yellow and red Atg8 dots in a ratio of 55% (red/total number of dots). In synjRQ there is a very significant reduction in the number of Atg8 dots (as we previously also showed with Atg8mCherry) (Figure R10 and Figure S2). Despite the low Atg8 dot counts in synjRQ we did assess the number yellow and red dots. We find a ratio of red over total Atg8 dots in synjRQ (57%) that is very similar to the ratio we measured in controls (Figure R10 and Figure S2). Hence, although very few Atg8 positive autophagosomes form in synjRQ mutants, the few ones that do form appear to mature similarly to autophagosomes in controls. These data are consistent with the idea that the block in autophagosome formation in synj mutants occurs early, before Atg8 recruitment.

Figure R10, added to Figure S2. Flux assay using GFP-mcherry-Atg8 reveals again a very low Atg8 dot number in synjRQ.

(A-C) Live imaging of starved NMJ boutons of Control (D42-Gal4>Atg8GFP-mCherry) (A-A”) and synjRQ (B-B”) also expressing Atg8GFP-mCherry. Full genotypes are included in supplemental section. Quantification of the total number of Atg8 (GFP+RFP and RFP alone) dots and the number of yellow (GFP+RFP) and red (RFP) Atg8 dots (C). Statistical analysis by t-test comparing each genotype individually, ***p<0.001, n≥7 larvae and n≥28 NMJs per genotype. Error bars represent the SEM, scale bar 5 µm. Note that in synjRQ, ass already seen with the mCherry-Atg8 construct a severe reduction in Atg8 dots is observed and that when an Atg8 dot is observed in synjRQ this will be degraded as controls.

4. Besides GFP-Atg18a, the authors should show the accumulation of PI3P in Synj-RQ mutant.
We have worked hard on assessing PI(3)P independently of Atg18aGFP and expressed the 2xFYVE-GFP probe that has been used in the past to monitor PI(3)P. Sadly, under the many conditions we used, these results are not conclusive and we suspect there are a number of reasons
that have been difficult to overcome. First, endosomes are also enriched in PI(3)P and the validated 2xFYVE-GFP probe is derived from Hrs, an endosomal protein (Wucherpfennig et al. 2003). It is therefore possible that the 2xFYVE preferentially labels endosomes while having lower affinity for autophagosome membranes, labelling them more sparsely. Second, Atg18a may have higher affinity for autophagosome membranes enriched in PI(3)P (Atg18a probably binds the lipid as well as other proteins on autophagosomes in a ‘coincidence detection mechanism’) thus competing with the lower affinity 2xFYVE-GFP probe. Finally, as shown below, we surmise that not only PI(3)P accumulates but also PI(3,5)P2 further complicating the use of other probes. We hope the reviewer agrees that despite our efforts it is difficult to address this question.

We have nonetheless performed an experiment that further argues that both PI(3)P and PI(3,5)P2 accumulate and have included these results in the reworked manuscript. The Synj SAC1 domain is capable of dephosphorylating several substrates, including PI(3)P and PI(3,5)P2, both of which can bind with Atg18a (Krück et al. 2006; Obara et al. 2008; Watanabe et al. 2012; Baskaran et al. 2012). To provide independent evidence of PI(3)P and/or PI(3,5)P2 accumulating in synjRQ animals we used pharmacology to block the production of either PI(3)P or PI(3,5)P2 in synjRQ mutants and monitored the accumulation of Atg18aGFP.

We used two drugs that are known to block kinases that produce PI(3)P (Vps34, blocked by wortmannin – WM) or PI(3,5)P2 (PIKfyve, blocked by YM201636 – YM). We first applied these drugs during starvation to control animals that express Atg18aGFP. While Atg18aGFP accumulates in dots in controls that are starved, application of either drug blocks this effect and Atg18aGFP does not accumulate anymore, indicating that Atg18aGFP likely binds both PI(3)P and PI(3,5)P2 during autophagy at synapses (Figure R8 and S3).

In synjRQ mutants the number of Atg18aGFP dots is always upregulated. However, when we starve the larvae in the presence of either WM or YM, the number of Atg18aGFP is lower than in untreated starved synjRQ mutants. These data suggest that both PI(3)P and PI(3,5)P2 accumulate and are likely substrates for Atg18a in synjRQ mutants (Figure R8 and S3).

5. It remains unclear on how Synj-RQ affected autophagosome formation. Can Atg18 knockdown or reduce the level of PI3P suppress Synj-RQ induced neurodegeneration in Fig5?

We agree with the reviewer that this is an interesting prediction that is testable. Atg18a accumulates in synjRQ mutants and genetically removing one copy of atg18a could possibly rescue (partly) the observed phenotypes at the level of starvation induced survival (we cannot remove both copies of atg18a because complete removal of atg18a is lethal).

We find that synjRQ; atg18a/+ animals on complete starvation live significantly longer than synjRQ animals, in line with the expectation (Figure R6). The problem here is that atg18a/+ animals alone live longer than the controls on complete starvation, making the results complicated to interpret. We also wanted to address neurodegeneration of dopaminergic neurons, but the heterozygous atg18a animals have a significantly lower number of dopaminergic neurons, making also the interpretation of this phenotype in this genetic background difficult. We hope the reviewer agrees it is better at this stage to not include these data in the main manuscript but we de present the starvation data here to the reviewers (Figure R6).

Additional References:
The EMBO Journal Peer Review Process File - EMBO-2016-95773


2nd Editorial Decision 23 February 2017

Thanks for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the three original referees and their comments are provided below. As you can see they appreciate the introduced revisions and support publication here.

Referee #2 has one last suggestion and that is to look at LC3 puncta or LC3 lipidation in the iPSC derived neuron experiment. This experiment should be straightforward to do and not involve too much additional work - let me know if I am mistaken and we can discuss further.

That should be all - looking forward to seeing the final version!

REFEREE REPORTS

Referee #1:
The authors performed a number of new experiments and satisfactorily addressed all my concerns.

Referee #2:
The authors have been very responsive and addressed most of my concerns. There is only one point that needs to be better addressed.

The authors now provide data showing that iPSC derived neurons with the Synj1 mutation have a greater number of WIP1-2 puncta. This is an important validation of the fly work, but it lacks one key element: is autophagy reduced as a result of this mutation? In this referee's opinion, it is not enough to show an increase in WIP2 puncta to demonstrate an impairment in autophagy. In fact this phenotype may potentially reflect an increase in autophagy. At the very least, the authors should assess autophagy flux with starvation in the presence or absence of lysosomal blockers (chloroquine, bafilomycin A1 or a lysosomal protease inhibitor), examining LC3 puncta (by IF) or LC3 lipidation (by western). This referee is very sensitive to the tremendous amount of work the authors have done, but also feels that this important point has not been addressed in depth during the first round of revision.

Referee #3:
The authors have addressed my concerns and added several new data to strengthen their study. I recommend it for publication.

2nd Revision - authors' response 25 February 2017

Referee #1
The authors performed a number of new experiments and satisfactorily addressed all my concerns.
Referee #2:

The authors have been very responsive and addressed most of my concerns. There is only one point that needs to be better addressed.

The authors now provide data showing that iPSC derived neurons with the Synj1 mutation have a greater number of WIPI-2 puncta. This is an important validation of the fly work, but it lacks one key element: is autophagy reduced as a result of this mutation? In this referee's opinion, it is not enough to show an increase in WIP2 puncta to demonstrate an impairment in autophagy. In fact this phenotype may potentially reflect an increase in autophagy. At the very least, the authors should assess autophagy flux with starvation in the presence or absence of lysosomal blockers (chloroquine, bafilomycin A1 or a lysosomal protease inhibitor), examining LC3 puncta (by IF) or LC3 lipidation (by western). This referee is very sensitive to the tremendous amount of work the authors have done, but also feels that this important point has not been addressed in depth during the first round of revision.

We recognize the importance of validating additional autophagy markers in the iPSC neurons derived from SYNJ1 RQ patients but there are several problems: (1) We have immunolabeled these differentiated neurons with anti-LC3 repeatedly but have failed to obtain reliable labeling. The alternative would be to express a tagged LC3 but that requires generating virus (+controls) which we do not have at the moment. (2) Westerns are in our view much less useful because we are looking at synapse specific events that are lost when preparing whole cell lysates. (3) We do not have these iPS cells in culture and a new differentiation will take several months to complete. We understand the reviewer’s question, but would like to stress that we have done all these experiments (flux assay, chloroquine) in vivo in flies and I am concerned that postponing the publication of this work by this much time would not serve the scientific community. Finally, we also want to emphasize the mechanistic insight provided here will feed the scientific community in further understanding the link between Synaptojanin and Parkinson’s disease.

Referee #3:

The authors have addressed my concerns and added several new data to strengthen their study. I recommend it for publication.
**Reporting Checklist for Life Sciences Articles (Rev. July 2015)**

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal’s authorship guidelines in preparing your manuscript.

### A. Figures

1. **Data**

   The data shown in figures should satisfy the following conditions:
   - The data were obtained and processed according to the cited method and are presented to reflect the results of the experiments in an accurate and unaided manner.
   - Figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
   - Graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
   - If n < 3, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
   - Source data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation.

2. **Captions**

   Each figure caption should contain the following information, for each panel where they are relevant:
   - A specification of the experimental system investigated (e.g. cell line, species name).
   - The assay(s) and method(s) used to carry out the reported observations and measurements.
   - An explicit mention of the biological and chemical entity(ies) that are being measured.
   - An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
   - The exact sample size (n) for each experimental group/condition, given as a number, not a range.
   - A statement of how many times the experiment shown was independently replicated in the laboratory.
   - Definitions of statistical methods and measures:
     - Common tests, such as t test (please specify whether paired or unpaired), simple p values, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section.
     - Are tests one-sided or two-sided?
     - Are there adjustments for multiple comparisons?
     - Exact statistical test results; e.g., P value ≤ x but P value < e.
     - Definitions of “center value” as median or average.
     - Definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

### B. Statistics and general methods

1. a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?
   - Yes, we did not pre-determine a “effect size” and therefore did not calculate power to estimate needed sample size.

1. b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.
   - Yes, no animals were used in this study.

2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?
   - Yes, no samples/animals were excluded in the analyses in this study.

3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.
   - Yes, no group allocation was used in this study.

4. Were any steps taken to minimize the effects of subjective bias during group allocation or/and while assessing results (e.g. blinding of the investigator)? If yes please describe.
   - Yes, ensuring results were blinded. Extra words: quantified data. This is stated in the materials and methods section.

5. a. For animal studies, include a statement about randomization even if no randomization was used.
   - Yes, no animals were used in this study.

5. b. Were any steps taken to ensure that the data were analyzed with group allocation or/and while assessing results (e.g. blinding of the investigator)? If yes please describe.
   - Yes, ensuring results were blinded. Extra words: quantified data. This is stated in the materials and methods section.

6. a. For animal studies, include a statement about blinding even if no blinding was done.
   - Yes, no animals were used in this study.

6. b. For every figure, are statistical tests justified as appropriate?
   - Yes, all tests used were stated in every figure legend if necessary.

7. Were the data from the experiments (e.g., normal distribution) describe any methods used to assess if this was true?
   - Before every statistical testing, the data was analyzed for normal distribution using a Shapiro-Wilk Pearson-Chisq test and descriptive statistics where performed. This is stated in the materials and methods section under Statistics.

8. a. If there is an estimate of variance within each group of data?
   - Yes, we used each time the SEM (standard error to the mean) reported in every figure legend if necessary.

9. b. The data variance among the groups that are being statistically compared?
   - Yes, an estimate of variance was used.
D- Animal Models

1. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

2. For experiments involving the volunteers, include a statement of compliance with ethical regulators and identify the committee(s) approving the experiments.

3. We recommend consulting the ARRIVE guidelines (see link list at top right) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under ‘Reporting Guidelines’. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.

E- Human Subjects

5. Identify the committee(s) approving the study protocol.

6. Include a statement confirming that informed consent was obtained from all subjects and that the experiments were performed in accordance with the principles set out in the WHA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

8. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

10. We recommend consulting the ARRIVE guidelines (see link list at top right) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under ‘Reporting Guidelines’. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.

F- Data Accessibility

16. Provide accession codes for deposited data. See author guidelines, under ‘Data deposition’.

17. Data deposition is a public repository is mandatory for:
   a. Protein, DNA and RNA sequences
   b. Microstructural structures
   c. Crystallographic data for small molecules
   d. Functional genomics data
   e. Proteomics and molecular interactions

18. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the Human data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under ‘Expanded View’ or in unstructured repositories such as Dryad (see link list at top right) or FigShare (see link list at top right).

19. Access to human data and genetic data should be provided with a few restrictions as possible while protecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access controlled repositories such as Biorxiv (see link list at top right) or GSA (see link list at top right).

20. If an item is available, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.

G- Dual use research of concern

22. Cite your study in the lead-in that uses research technologies! Please check biosafety guidelines (see link list at top right) and list of select agents and toxins (AMRA/DCG) (see link list at top right). According to our biosafety guidelines, provide a statement only if it could.