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TBK1 suppresses mutant HTT induced toxicity and pathology in models of Huntington's disease

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1st Editorial Decision 9th Apr 2020

Dear Hilal,

Thanks for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you, but I have now received the needed input on your study.

Your manuscript has now been seen by three referees and their comments are provided below. As you can see from the reports, while referee #2 and 3 are very supportive of the study referee #1 is less so. I have taken a careful look at the comments raised and also consulted further with referee #2 and 3. Referee #1 questions the use of exon 1 models of HD and the role of toxic aggregates in HD. I accept the opinion of referee #1, but there are also other viewpoints on these issues. I have discussed the raised issues further with the two other referees and they agreed that the analysis provides valuable and important new insight.

We are interested in considering a revised version should you be able to address the concerns raised by referee #3 and where appropriate by referee #1. This includes a better discussion of previous work and more work to substantiate the key findings. I am happy to discuss the raised points further and maybe it would be most helpful to do so via phone or skype. I will contact you in the next few days to discuss this further. I am also aware that with the current Covid-19 situation and lab closures that carrying out experimental revisions is not so straightforward. There is no problem on our end to extend the revision time as much as needed to carry out the needed revisions. We can discuss this further in the call.

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:
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I thank you for the opportunity to consider your work for publication. I look forward to discussing your revisions with you.

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

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- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Referee #1:

This study is looking at the modification of serine 13 in huntingtin within the N17 domain which is known to modulate huntingtin activity and localization. From an in vitro screen, they identified TANK-binding kinase, TBK-1, and suggest this modification is triggering autophagic clearance of mutant huntingtin.
The manuscript is a good example of confirmation bias. The mechanism is predicted on the concept of toxic aggregates in HD, which was a major focus in HD for 20 years and yielded multiple pathways for which none have been validated in actual disease. The concept that protein aggregates cause HD is outdated, and limited to systems where 97% of the huntingtin protein is eliminated, and the remaining fragment is massively overexpressed with either a synthetic allele or genetic allele taken from extreme outlier juvenile onset HD. CAG40-50 encompasses >80% of clinical HD, yet these fragment model systems exceed this considerably, and then driven by a powerful viral promoter. At no point does this study look at real, endogenous huntingtin, but unfortunately remains in systems where small fragments only are used, with massive overexpression. This is for good reason, because recent studies on iPSC and other genetically accurate human cell lines in which HD phenotypes can be detected, express these phenotypes

without the presence of any protein aggregation. HD brain data showing no correlation between sites of pathology and aggregate presence are over 15 years old.
In 2015, GWAS studies in HD to define pathways that radically alter the age of onset of HD did not define a single pathway of protein homeostasis. For the pathways that are affected, none were ever defined in HD exon1 models.
In 2019, the single most effective modifier of HD was the presence of an alternative codon in the CAG tract, for which the exact same protein sequence is produced. Two groups independently concluded that this allele was even more important that the CAG length, hence polyglutamine in the huntingtin protein. The point is, the field moved away from the toxic aggregate hypothesis over a decade ago, and concepts of selective autophagy to remove a protein are naïve.

This work would be better suited for an HD specialty journal.

My summary of some the major issues:

1. Exon1 models of HD are outdated and no longer considered valid.
2. The entire data set avoids any control of protein stoichiometry. This is critical for any signaling study and has been proven over and over in signaling since the early 90s.
3. The entire manuscript avoids any study of full-length huntingtin-not a single western is above 60Kda.
4. HEK293T cells are massive overexpression of fragments, by molarity, 2000-3000X or more over endogenous. All this does is reproduce the same artifacts of protein concentration from in vitro experiments. These lines have no effective P53 (which regulates huntingtin), and are notorious for irreproducibility.
5. The data representation is poor. Many journals no longer accept bar graph data, and cutting off the lower half of error bars for aesthetic reasons is not scientifically valid. Here is why: https://www.jbc.org/content/292/50/20592.full
6. There are no experiments to define selective autophagy.
7. At no point in the data were typical clinical alleles of mutant huntingtin from actual disease used.
8. They needed controls of any other aggregate-prone protein to validate these readouts are even specific to a small fragment of HD.This control is missing from all exon1 model data in the press to date.
9. Improper use of statistics. No mentions of tests used in figure legends, but it looks like T-tests were used where ANOVA was required, and it's impossible to know if the data was normal as presented (back to point 5).
10. The concept that alanine substitutions are not the same as unmodified serines is repeatedly stressed, but surely this is common knowledge in 2020.
11. The model is far too speculative for this data. There were no aggregate ultrastructure data to define fibrils, oligomers, inclusions, etc. This was all taken from old theories.

Referee #2:

The study by Hegde et al. helps to resolve a longstanding important question in the Huntington's disease field regarding the endogenous kinase(s) that can mediate the phosphorylation of serine 13 and/or serine 16 in the Huntingtin (HTT) N17 domain that results in neuroprotective effects. A study in 2009 (PMID: 20064390) showed that BAC transgenic mouse models of HD with phosphomimetic mutations (S13D, S16D) at these sites, but not those with phospho-blocking mutations (S13A, S16A), can abolish the toxicities of mHTT in vivo (behavioral deficits, brain atrophy) and mHTT aggregation. Subsequent biochemical and cell biological studies by others in

the field, including those from Ray Truant lab and Leslie Thompson/Joan Steffan lab, by and large support a model that the phosphomimetic mutations or phosphorylation at S13 and S16 can suppress mHTT aggregation and reduce its cytotoxicity. Thus, identification of a kinase that can phosphorylate wildtype or mutant Huntingtin (mHTT) at these sites would represent a significant step forward towards a PTM-targeting therapeutic strategy for the HD field. A prior study implicates IKK as a kinase for HTT S13 and S16 (PMID: 20026656), but another study provided seemingly contrary findings (PMID: 21623356). In this context, the current study represents by far the most definitive demonstration of a S13 kinase for HTT. The authors performed an unbiased in vitro screen of about 300 kinases to identify TBK1 along with IKKb as kinases for N17 at S13 and S16 residues. Subsequently, they used an elegant series of biochemical and cell biological experiments to show that TBK1 overexpression can increase serine 13 phosphorylation on wildtype HTT and mHTT in vitro and in cells (including neurons), and unlike IKKb, such S13 phosphorylation is not diminished with mHTT polyQ expansion. Interestingly, increased S13 phosphorylation by TBK1 leads to a reduction of HTT/mHTT levels. The study probed several mechanistic aspects of TBK1-HTT interactions. It showed that TBK1-mediated HTT phosphorylation drives its nuclear translocation and reduction of mHTT aggregation. Moreover, TBK1 appears to act at the monomers, not aggregates, of mHTT to reduce its level and aggregation. Additionally, the authors discovered an unexpected effect of TBK1 in the reduction of soluble HTT that is primarily dependent on the increase of autophagic flux, and only secondarily dependent on the phosphorylation of mHTT at S13. Finally, the study used a C. elegans model of mHTT toxicity to show that overexpression or knockdown of TBK1 can opposingly regulate mHTT toxicity and aggregation, which is consistent with the proposed mechanism.

Together, this is a high quality and exciting study with compelling data to help resolving a longstanding important question in the HD field, and the findings are novel and mechanistically and therapeutically informative. Thus, it is suitable for publication in EMBO.

Referee #3:

Review of "TBK1 suppresses mutant HTT induced toxicity and pathology in models of Huntington's disease" by Hedge et al., 2020, submitted to EMBO Journal.

Huntington's Disease (HD) is caused by a mutation encoding an expansion of a polyglutamine (polyQ) repeat within the protein Huntingtin (HTT), resulting in mutant HTT accumulation in neuronal aggregates, and cellular toxicity with striatal neurodegeneration. The authors of this manuscript under review for EMBO Journal demonstrate that TBK1, a family member of the IKK and IKK-related kinases, can phosphorylate serine (S) 13 of HTT as has been previously shown for the IKKalpha and IKKbeta catalytic subunits of the IKK complex (PMID: 20026656, PMID: 31088970). Similarly, this manuscript shows that this phosphorylation event is associated with HTT autophagic degradation, which may be linked to its function as a scaffold for selective autophagy (PMID: 25385587, PMID: 25686248). Despite sharing high levels of homology (TBK1 is 27% identical to both IKKalpha and IKKbeta) and some degree of functional redundancy, TBK1, IKKalpha and IKKbeta perform distinct cellular roles and this manuscript therefore does impart important novel information to the community of scientists working on HD pathogenesis, particularly as HTT interacts with two autophagy receptor proteins, Optineurin and SQSTM1/p62, which are activated to bind ubiquitinated autophagic cargo through direct phosphorylation by TBK1 (PMID: 29867991, PMID: 25972374) - the current manuscript may therefore suggest that TBK1 is a master activator of autophagy in which HTT, SQSTM1/p62 and Optineurin function. IKKbeta knockdown in vivo in

mice has previously been shown to significantly reduce, but not completely eliminate HTT S13 phosphorylation (PMID: 31088970), leaving room for other kinases in vivo, although in the present manuscript, mouse cortical tbk1-/- tissue shows total HTT accumulation with higher HTT pS13 levels than tbk1+/+ tissue, suggesting TBK1's major function in vivo in brain tissue may be that of activating autophagy in general to eliminate phosphorylated HTT, and not as a primary HTT S13 kinase in vivo (manuscript figure S3IJ).

This manuscript demonstrates that TBK1, pulled out by the authors in a screen of 300 kinases, can function to phosphorylate S13 and S16 of wt HTT peptide and fragments in vitro, although the authors do not have a pS16 antibody that works in their hands for cell culture or tissue, so this body of work is largely examining phosphorylation of HTT S13. Similarly, they demonstrate that overexpression of TBK1 but not kinase dead TBK1 (KD) can increase S13 phosphorylation in non-neuronal (human epithelial embryonic kidney HEK293T) cell culture. The authors use overexpression of TBK1 (or TBK1 KD) and HTT to examine its specificity and efficiency for phosphorylating full-length and N-terminal fragments of HTT in HEK293T, primary neuronal culture and in C. elegans in vivo, and examine the effect of TBK1 on HTT abundance and cellular localization. They also find that an activation of autophagy by TBK1 may reduce HTT aggregation through a reduction in HTT abundance due to increased autophagic flux, suggesting that a therapeutic strategy to activate TBK1 and autophagy may be useful to treat HD.

This manuscript has many new important findings relevant to HTT S13 phosphorylation and clearance that are novel and convincing, and does suggest a new therapeutic target, TBK1, for HD drug development. I like this work very much and it is well written and engaging. It is disturbing, however, that in several instances the claims are not appropriately discussed in the context of earlier literature. As in the abstract, the claim of "the lack of natural kinases that regulate N-terminal phosphorylation" is false, as IKKbeta was previously shown to phosphorylate HTT S13 in vitro, in cell culture, and in vivo (PMID: 20026656, PMID: 31088970). Note that reduction of IKKbeta levels in both wt or in R6/1 HD mouse striatum significantly increased levels of full-length mouse HTT and reduced levels of S13 phosphorylation on full-length mouse HTT in vivo. IKKbeta knockdown also further impaired HD behavior and increased neurodegeneration and microglial activation in R6/1 mice. These results for IKKbeta are in the same vein as the results being described in this manuscript for TBK1 involving HTT S13, but are not discussed. I also find the following statement dismissive of other relevant literature: "We recently showed that phosphorylation of HTT at S13 and/or S16 inhibits mutant HTTex1 aggregation in vitro (Deguire et al., 2018) and increases its conformational flexibility (Daldin etal., 2017). A direct comparison of the effect of phosphomimetics and bona fide phosphorylation at these sites also showed that the phosphomimetics (S13D/S16D) only partially reproduced the effect of phosphorylation on aggregation and did not reproduce the effect of single and double phosphorylation at these residues on the helical conformation of N17 (Deguire et al., 2018)." I do not agree that work with phosphomimetics from several groups is completely irrelevant and should be dismissed. In fact, in 2009 S13/16D HTT peptide showed in vitro reduced aggregation (PMID: 20064390) similar to Deguire 2018, and HTT S13/16D exon 1 fragment abundance in cell culture was found to be reduced (PMID: 20026656), which would support the findings of this manuscript under review. Within their manuscript, the authors include S13/16A phosphoresistant mutation, but not S13/16D phosphomimetic mutation in their studies, inclusion of which might have informed their work with TBK1-mediated HTT phosphorylation in their system in cells. In the context of dopaminergic neurons in adult Drosophila brain (PMID: 28934390), mimicking phosphorylation of S13 (S13D) within mutant HTT exon 1 protein increases aggregation (the opposite of what has been found in vitro by the authors and others), suggesting that in vivo phosphorylation of S13 may create an oligomerized autophagic clearance intermediate. This may be activated by IKKbeta- or TBK1-mediated HTT phosphorylation and cleared with the induction of

autophagic flux activated by both kinases - this is not considered in the present manuscript. Oligomerization has been found to guide pathway choice between proteasomal and autophagic degradation (PMID: 28504708). As the authors have examined in vitro effects of phosphorylation of N17 to reduce aggregation, they need to also consider that in vivo, TBK1 and IKKbeta may both increase the formation of oligomerized autophagic clearance intermediates through phosphorylation of HTT S13. I therefore feel that the authors need to represent earlier literature more fairly, even though all these paper were indeed cited in the text.

I have some questions regarding several of the figures, please see numbered comments below:

1. Figure 1DE and S2AB: What antibody is being used to detect HTT here? I would appreciate the authors putting the anti-HTT antibody used for each figure into the figure legend. As far as I can tell, 1D may use anti-eGFP? HTT exon 1 protein is greatly stabilized by fusion to a C-terminal GFP tag, and the GFP tag can be post-translationally modified impacting its clearance (PMID: 15064418). Does this eGFP tag impact the clearance of 16Q and 72Q HTT exon 1 protein in their hands and what impact does that have on the levels of total HTT protein that are changing in this experiment?

Although Origene's Myc-tagged IKKbeta and TBK1 constructs appear to be equivalently expressed by Western with anti-Myc, this does not mean that they are equally active with Myc tag fusion. Also, IKKalpha, IKKbeta and IKKgamma often work together within the IKK complex, while TBK1 is simply a dimer of identical subunits - the authors don't have all 3 IKK subunits in their in vitro or cell culture systems, which might impact the relative data intensity when comparing IKKbeta to TBK1 activity (Figures 1DE and S2AB). Note it has previously been published that both IKKalpha and IKKbeta catalytic subunits can directly phosphorylate HTT S13 on wt and mutant HTT exon 1 protein in vitro, and overexpression of IKKalpha in cell culture also increases phosphorylation of HTT S13 and S16 (PMID: 20026656). In addition, the IKK complex regulatory subunit IKKgamma has been shown to directly interact with HTT's proline-rich region adjacent to N17 (PMID: 15371500) - the lack of the regulatory subunit in the in vitro kinase assay may slow recruitment as in S2B. Without the entire IKK complex in the authors' studies, I believe it is impossible to suggest that TBK1 is a stronger S13 kinase than IKKbeta functioning within context of the IKK complex, which is present in vivo. What the authors can say is that they both activate phosphorylation of HTT S13 in their hands.

2. Figure 2:
2A: Is this a Western using anti-eGFP antibody to detect HTT? Note that there is residual pS13 activity in the wt and the T3A 16Q with TBK1 KD overexpression - there is another active kinase in the HEK293T cells - how about IKK complex?
2C: What antibody is being used for anti-HTT? Quantitation by Singulex assay from the authors' previous 2020 paper (PMID: 31677786) would suggest MW1/2B7 was used in the Singulex assay? 2B7 is a monoclonal antibody with a small epitope including HTT lysine K9, which becomes acetylated (potentially destroying the 2B7 epitope) with S13 phosphorylation (PMID: 20026656). Therefore 2B7 may not detect the modified species cleared by autophagy - it may not be a total HTT antibody and should not be used to quantitate relative levels of pS13. I note that TBK1 overexpression in the context of HTT N548 55Q does not reduce the fragment - is this because the authors are detecting with 2B7? Note good levels of p13 phosphorylation again without TBK1 overexpression - is this IKK?
2EF: What antibody is being used for anti-HTT - 2B7? The legend says the authors are comparing full-length 23Q vs. 48Q HTT, but the figure shows S13/16A? Are these wt 48Q and S13/16A 48Q, without 23Q in the figure? Why are levels of TBK1 KD so low in this experiment? - it is not a control

for wt TBK1 in this panel. Note that if the authors have high levels of pS13 in the wt 48Q this might reflect upregulation of IKK activity with polyQ expanded HTT expressed consistent with the work of Ali Khoshnan/Paul Patterson from 2004 PMID: 15371500. Once again the Singulex assay 2F does not appear to be validating the Western in 2E.
2G: What antibody is being used for anti-HTT detection - I see that D7F7 was used for the IP of endogenous wt HTT in rat primary striatal neurons, and here authors are observing reduced levels of full-length HTT and increased HTT phosphorylation with TBK1 overexpression. This figure is nice, but the antibodies used need to be written in the legend. Is LV GFP expression increasing levels of full length wt HTT over LV TBK1 KD, suggesting a blockage of HTT autophagic clearance by GFP?

3. Figure 3: Is the antibody detecting the HTT construct on the Western anti-eGFP?
3A: Again, what impact does eGFP have on abundance of these exon 1 proteins (as eGFP fusion itself stabilizes HTT exon 1 protein), and can IKKbeta and TBK1 directly be compared based on overexpression of myc-tagged constructs without co-overexpression of IKKalpha and IKKgamma with IKKbeta? It does look like IKKbeta and TBK1 are reducing HTT levels both in the whole cell extract and in aggregates independent of phosphorylation of S13/16. Might S13/16D have done the same without the kinases - is expression of the kinase essential here for increased clearance and reduced aggregates - what is the contribution of phosphorylation alone to abundance and aggregation? I think the authors have done themselves a disservice by not using S13/16D mutants for figure 3, although technically the data is solid. Figure S4B, note that 72Q with TBK1KD has more nuclear localization than 16Q with TBK1KD - is this significant and does this reflect endogenous activation of IKK with mutant HTT expression as previously described (PMID: 15371500)?

4. Figure 5: Is the anti-HTT antibody anti-YFP in these experiments? Isn't Unc-51 generally considered an Atg1/ULK2/ULK1 homolog, not a TBK1 homolog? The IKK complex and TBK1 are not conserved in C. elegans, and there is no "HTT" - N17 is not present or conserved. TBK1 is not greatly impacting levels of transiently transfected soluble N513 fragment but does reduce aggregates - does YFP fusion stabilize N513 in C. elegans? Is TBK1 in this case actually clearing aggregates, and not soluble oligomers? pS13 does not appear unstable in this system - is the autophagic mechanism for human N513 not precisely conserved in C. elegans? TBK1 is highly related to IKK-epsilon (49% identical), so it does make sense that C. elegans IKK-epsilon is the endogenous kinase available to phosphorylate human HTT S13 in this system, but it does not impact stability of soluble or insoluble human N513. Is endogenous C. elegans IKK-epsilon responsible for the basal pS13 activity in 5D? Does human IKK-epsilon regulate HTT pS13 phosphorylation in HEK293T cells as well? Based on homology I suspect it does. Is it a relevant pS13 kinase in vivo like IKKbeta, as TBK1 may not be based on figure S3I? Was it one of the 300 kinases tested in the in vitro kinase original screen?

5. Figure 6 and S6: TBK1 is well characterized to activate autophagy, and it is also well defined that HTT aggregate numbers are reduced with activation of autophagy, although it is good to see in the authors' system.

6. Figure 7: Figure 7B bottom, and 7D label is incorrect - the delta 690-713 is not written in so it is confusing.

7. Figure S3: A panel from S3A with mass spec data is occluding figure S3CDFG - a formatting issue with the figure. What antibody is used for S3E (I am assuming anti-eGFP is used for S3CD)? Figure S3GH has controls on the end and everything else seems higher, even with titration of drugs. I find this figure difficult to believe. What total anti-HTT antibody is used for S3IJ? For S3EF and IJ, what

is the ratio of pS13/total HTT? I can see that full-length HTT accumulates with TBK1 knockdown, but more pS13 reactivity is present as well. Does the ratio of pS13/total HTT drop with TBK1 reduction?

8. Page 1 of manuscript: I do not believe the manuscript shows that "TBK1-mediated neuroprotective effects are due to phosphorylation-dependent inhibition of mutant HTTex1 aggregation" - it shows that HTT abundance is reduced with pS13, and that autophagy is activated thereby reducing abundance (which both may result in reduced aggregates, but not a direct inhibition of aggregation within N17, so perhaps the way this is written is misleading).

9. Page 5: Ochaba et al 2019 recently showed that IKKbeta increased HTT S13 phosphorylation in HD and wt control mice in vivo.

10. Page 11, line 5, Marc Diamond's paper should be included in the citations here PMID: 23319588.

11. Page 12, line 2, Saudou's 2007 paper does not deal with S13/S16 - it only deals with S1181/S1201 which are cdk5 sites. Gu et al, 2009 PMID: 20064390 is the missing reference for here.

Responses to referee's comments and questions

Referee#1:

This study is looking at the modification of serine 13 in huntingtin within the N17 domain which is known to modulate huntingtin activity and localization. From an in vitro screen, they identified TANK-binding kinase, TBK-1, and suggest this modification is triggering autophagic clearance of mutant huntingtin.

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Response: The exon 1 model is still very relevant. Over the last few years, it has been shown that exon 1 of the huntingtin gene (*HTT*) does not always splice to exon 2, resulting in the production of a small polyadenylated transcript that encodes the exon 1 HTT protein (Sathasivam, Neueder et al., 2013). The longer the CAG repeat, the more of this incomplete splicing occurs. Every knock-in mouse model as well as those transgenic for the full-length human *HTT* gene (e.g. YAC128) produce an exon 1 HTT protein (Franich, Hickey et al., 2019, Sathasivam et al., 2013). Disease onset and progression in knock-in mice correlates with the level of incomplete splicing, of the exon 1 HTT protein and the appearance of aggregates (Franich et al., 2019). This incomplete splicing event occurs in HD patients brains and cell lines (Neueder, Landles et al., 2017).

It was shown in 2003 that somatic expansion can result in CAG repeats in the brains of HD patients of several hundred and up to 1000 CAGs. The identification of genetic modifiers that act to modulate somatic instability of the CAG repeat, has brought this process into sharp focus. We do not know what the pathogenic length of a CAG repeat in HD patient brains might be, but it is quite likely that it is longer than the 40-50 CAGs found in blood, and means that the mouse models expressing CAG repeats from 80 to 200 are likely to have disease relevance.

This is for good reason, because recent studies on iPSC and other genetically accurate human cell lines in which HD phenotypes can be detected, express these phenotypes without the presence of any protein aggregation. HD brain data showing no correlation between sites of pathology and aggregate presence are over 15 years old.

Response: It is premature to make this statement. iPSC models have yet to be completely characterized. Phenotypes need to be replicated in studies with isogenic lines and the number of biological replicates to give good statistical power. Sensitive approaches to look for aggregate species have not been performed.

In 2015, GWAS studies in HD to define pathways that radically alter the age of onset of HD did not define a single pathway of protein homeostasis. For the pathways that are affected, none were ever defined in HD exon1 models.

Response: This is not true. In 1999 Anne Messer showed that if you crossed the R6/1 transgene (exon 1) onto an Msh2 null background, somatic instability of the CAG repeat was prevented. Over the next 15 years, various groups had shown that knocking-out components of the mismatch repair pathway in exon 1 or knock-in mouse models of HD prevented somatic instability. Therefore, it was very exciting in 2015, and later in 2019, when the GEM-HD consortium showed that many of the genetic modifiers of HD are mismatch repair genes. The functional analysis had already been done in mice.

The consequence of somatic expansion is that longer CAG repeats result in more incomplete splicing of the *HTT* gene, and this produces more of the exon 1 HTT protein. One current hypothesis states that

the genetic modifiers are working by decreasing the level of the toxic exon 1 HTT protein and it is this, that results in later disease-onset or slower progression.

In 2019, the single most effective modifier of HD was the presence of an alternative codon in the CAG tract, for which the exact same protein sequence is produced. Two groups independently concluded that this allele was even more important that the CAG length, hence polyglutamine in the huntingtin protein.

Response: This statement would only be relevant if the mutation was acting at the DNA level, which no-one believes to be the case. The point is that, for example, $(CAG)_{43}$ results in disease onset earlier than $(CAG)_{41}CAACAG$, although these tracts encode the same number of glutamines. However, the pure CAG tract results in greater levels of somatic instability, and longer CAG repeats brain regions e.g. striatum. Therefore, the polyglutamine tracts in brain regions from the $(CAG)_{43}$ are likely to be longer than from $(CAG)_{41}CAACAG$. This 2019 paper did not negate the contribution of aggregates to the toxicity. Indeed, it states the following *"it is important to state that this does not preclude a role for polyglutamine in HD, since it remains a candidate for the toxicity driver that acts after the CAG repeat length has determined the timing of onset."*

The point is, the field moved away from the toxic aggregate hypothesis over a decade ago, and concepts of selective autophagy to remove a protein are naïve.

Response: This is not a view held by most people in the field.

This work would be better suited for an HD specialty journal.

My summary of some the major issues:

1. *Exon1 models of HD are outdated and no longer considered valid.*

Response:

- We believe that exon 1 model is still useful to study huntingtin because, expression of pathogenic HTT exon1 was sufficient to induce HD-like features including aggregates formation and toxicity in mice (Mangiarini, Sathasivam et al., 1996), cells (Scherzinger, Sittler et al., 1999) and C. elegans (Wang, Lim et al., 2006).
- In our manuscript we used other models based on longer HTT fragments, including HTT full-length (Fig 2E-H)), 586 (Fig 4 B, D) 548 (Fig 2 C, D) and HTT 513 worm model (Fig 5 A-E) all of which show increased HTT S13 phosphorylation upon TBK1 expression.

2. *The entire data set avoids any control of protein stoichiometry. This is critical for any signaling study and has been proven over and over in signaling since the early 90s.*

Response: We are not clear what the referee is referring to here and would appreciate further clarification on this point.

3. *The entire manuscript avoids any study of full-length huntingtin-not a single western is above 60Kda.*

Response: We respectfully disagree. We have assessed TBK1-mediated phosphorylation of exon1 and full-length HTT, see Fig 2E-H. In addition, we assessed the effect of TBK1 on models expressing longer HTT fragment, namely, HTT 586 (Fig 4 B, D) HTT 548 (Fig 2 C, D) and HTT 513 worm model (Fig 5 A-E), all of which show increased HTT S13 phosphorylation up on TBK1 expression.

4. *HEK293T cells are massive overexpression of fragments, by molarity, 2000-3000X or more over endogenous. All this does is reproduce the same artifacts of protein concentration from in vitro experiments. These lines have no effective P53 (which regulates huntingtin), and are notorious for irreproducibility.*

Response:

- We have used the HEK293 model overexpression model and verified the findings in a primary neuronal model of HD. In HEK 293 cells, we observed 5-20x overexpression using various plasmids, as measured by western blotting using the anti HTT MAB2166 antibody as represented in Fig R1.
- HTT exon1 expression in HEK293 results in cytosolic aggregates and is one of the most commonly used models of HTT aggregation and inclusion formation (Nguyen, Hamby et al., 2005) Fig.1, (Jiang, Wei et al., 2015) Fig.1 A, Fig 3H (Bersuker, Hipp et al., 2013) etc.
- In the manuscript, we also used primary striatal neuronal model and a worm model to confirm our key observations; namely,
  a) TBK1 expression increases the S13 phosphorylation of endogenous HTT in primary rat neurons (Fig 2 G)
  b) TBK1 expression suppresses neurotoxicity in primary striatal neurons transfected with HTTN586 82Q fragment (Fig 4 B, C).
  c) TBK1 expression increases the S13 phosphorylation suppresses toxicity in *C. elegans* model of HTTN513 128Q (Fig 5 D-G)

5. *The data representation is poor. Many journals no longer accept bar graph data, and cutting off the lower half of error bars for aesthetic reasons is not scientifically valid. Here is why: HTTps://www.jbc.org/content/292/50/20592.full*

Response: As recommended, we have now changed data representation of the bar graph to include the individual data points and both halves of the error bars.

6. *There are no experiments to define selective autophagy.*

Response:

- The role of TBK1 in selective autophagy is well documented in the literature.
  a) It was shown to phosphorylate autophagy receptors p62, NDP52 and OPTN and increase their binding affinity for polyubiquitin chains on damaged mitochondria, intracellular pathogens and the LC3 family proteins, thereby driving autophagy (Richter, Sliter et al., 2016, Wild, Farhan et al., 2011).
  b) It Phosphorylates LC3C which aids in maintaining a unidirectional flow of the autophagosome to the lysosome (Herhaus, Bhaskara et al., 2020).
- Therefore, TBK1 may activate ubiquitin-dependent autophagy of the substrates specific to OPTN, p62 and NDP52.

7. *At no point in the data were typical clinical alleles of mutant huntingtin from actual disease used.*

Response: We respectfully disagree. We have used a range of mutant HTT with different polyQ repeat lengths in different cellular models and in all cases TBK1 efficiently phosphorylated HTT
  a) HTT-FL 48Q (Fig 2 E)
  b) HTTN548 55Q (Fig 2 C).

8. *They needed controls of any other aggregate-prone protein to validate these readouts are even specific to a small fragment of HD. This control is missing from all exon1 model data in the press to date.*

Response: TBK1 expression has been shown to reduce the aggregation of SOD1 mutant G93A and G93C (Duan, Guo et al., 2019) (Korac, Schaeffer et al., 2013).
We also observed that TBK1 indeed reduced the soluble and aggregated forms of SOD1 G90A upon coexpression (For the benefit of the referee, see Fig R2)

Investigating the effect of TBK1 on the aggregation and clearance of several aggregation-prone proteins is interesting but is beyond the scope of this manuscript.

9. *Improper use of statistics. No mentions of tests used in figure legends, but it looks like T-tests were used where ANOVA was required, and it's impossible to know if the data was normal as presented (back to point 5).*

Response: We thank the referee for bringing this to our attention. We have used the students T-test as described in the materials and methods section. We now clearly specify the statistics used for each figure.

10. *The concept that alanine substitutions are not the same as unmodified serines is repeatedly stressed, but surely this is common knowledge in 2020.*

Response: We agree. However, despite the fact this concept is well-documented in the literature, all publications prior to our work still rely on such mutations to investigate the role of phosphorylation in regulating Htt biology, aggregation and toxicity.

11. *The model is far too speculative for this data. There were no aggregate ultrastructure data to define fibrils, oligomers, inclusions, etc. This was all taken from old theories.*

Response:

- This is a well-established model of HTT inclusion formation and has been characterized by multiple independent groups and methods, including super-resolution microscopy (Bauerlein, Saha et al., 2017, DeGuire, Ruggeri et al., 2018, Isas, Langen et al., 2015, Lin, Boatz et al., 2017, Menon, Pandey et al., 1998, Pandey, Isas et al., 2018, Warner, Ruff et al., 2017).
- We believe that a detailed characterization of the oligomerization and ultrastructural properties of HTT inclusions is a huge effort and beyond the scope of this manuscript.
- Indeed, it has taken us three years to achieve this goal and these efforts are part of a full manuscript that we would be happy to share. For the reference of the referee and editor, a copy of the manuscript is enclosed with this response.

Referee #2:

The study by Hegde et al. helps to resolve a longstanding important question in the Huntington's disease field regarding the endogenous kinase(s) that can mediate the phosphorylation of serine 13 and/or serine 16 in the Huntingtin (HTT) N17 domain that results in neuroprotective effects. A study in 2009 (PMID: 20064390) showed that BAC transgenic mouse models of HD with phosphomimetic mutations (S13D, S16D) at these sites, but not those with phospho-blocking mutations (S13A, S16A), can abolish the toxicities of mHTT in vivo (behavioral deficits, brain atrophy) and mHTT aggregation. Subsequent biochemical and cell biological studies by others in the field, including those from Ray Truant lab and Leslie Thompson/Joan Steffan lab, by and large support a model that the phosphomimetic mutations or phosphorylation at S13 and S16 can suppress mHTT aggregation and reduce its cytotoxicity. Thus, identification of a kinase that can phosphorylate wildtype or mutant Huntingtin (mHTT) at these sites would represent a significant step forward towards a PTM-targeting therapeutic strategy for the HD field. A prior study implicates IKK as a kinase for HTT S13 and S16 (PMID: 20026656), but another study provided seemingly contrary findings (PMID: 21623356). In this context, the current study represents by far the most definitive demonstration of a S13 kinase for HTT. The authors performed an unbiased in vitro screen of about 300 kinases to identify TBK1 along with IKKb as kinases for N17 at S13 and S16 residues. Subsequently, they used an elegant series of biochemical and cell biological experiments to show that TBK1 overexpression can increase serine 13 phosphorylation on wildtype HTT and mHTT in vitro and in cells (including neurons), and unlike IKKb, such S13 phosphorylation is not diminished with mHTT polyQ expansion. Interestingly, increased S13 phosphorylation by TBK1 leads to a reduction of HTT/mHTT levels. The study probed several mechanistic aspects of TBK1-HTT interactions. It showed that TBK1-mediated HTT phosphorylation drives its nuclear translocation and reduction of mHTT aggregation. Moreover, TBK1 appears to act at the monomers, not aggregates, of mHTT to reduce its level and aggregation. Additionally, the authors discovered an unexpected effect of TBK1 in the reduction of soluble HTT that is primarily dependent on the increase of autophagic flux, and only secondarily dependent on the phosphorylation of mHTT at S13. Finally, the study used a C. elegans model of mHTT toxicity to show that overexpression or knockdown of TBK1 can opposingly regulate mHTT toxicity and aggregation, which is consistent with the proposed mechanism.

Together, this is a high quality and exciting study with compelling data to help resolving a longstanding important question in the HD field, and the findings are novel and mechanistically and therapeutically informative. Thus, it is suitable for publication in EMBO.

Response: We thank the referee for acknowledging the importance and significance of our work and recommending the manuscript for publication in EMBO.

Referee#3:

Review of "TBK1 suppresses mutant HTT induced toxicity and pathology in models of Huntington's disease" by Hedge et al., 2020, submitted to EMBO Journal.

Huntington's Disease (HD) is caused by a mutation encoding an expansion of a polyglutamine (polyQ) repeat within the protein Huntingtin (HTT), resulting in mutant HTT accumulation in neuronal aggregates, and cellular toxicity with striatal neurodegeneration. The authors of this manuscript under review for EMBO Journal demonstrate that TBK1, a family member of the IKK and IKK-related kinases, can phosphorylate serine (S) 13 of HTT as has been previously shown for the IKKalpha and IKKbeta catalytic subunits of the IKK complex (PMID: 20026656, PMID: 31088970).

Similarly, this manuscript shows that this phosphorylation event is associated with HTT autophagic degradation, which may be linked to its function as a scaffold for selective autophagy (PMID: 25385587, PMID: 25686248).

Despite sharing high levels of homology (TBK1 is 27% identical to both IKKalpha and IKKbeta) and some degree of functional redundancy, TBK1, IKKalpha and IKKbeta perform distinct cellular roles and this manuscript therefore does impart important novel information to the community of scientists working on HD pathogenesis, particularly as HTT interacts with two autophagy receptor proteins, Optineurin and SQSTM1/p62, which are activated to bind ubiquitinated autophagic cargo through direct phosphorylation by TBK1 (PMID: 29867991, PMID: 25972374) - the current manuscript may therefore suggest that TBK1 is a master activator of autophagy in which HTT, SQSTM1/p62 and Optineurin function. IKKbeta knockdown in vivo in mice has previously been shown to significantly reduce, but not completely eliminate HTT S13 phosphorylation (PMID: 31088970), leaving room for other kinases in vivo, although in the present manuscript, mouse cortical tbk1-/- tissue shows total HTT accumulation with higher HTT pS13 levels than tbk1+/+ tissue, suggesting TBK1's major function in vivo in brain tissue may be that of activating autophagy in general to eliminate phosphorylated HTT, and not as a primary HTT S13 kinase in vivo (manuscript figure S3IJ).

This manuscript demonstrates that TBK1, pulled out by the authors in a screen of 300 kinases, can function to phosphorylate S13 and S16 of wt HTT peptide and fragments in vitro, although the authors do not have a pS16 antibody that works in their hands for cell culture or tissue, so this body of work is largely examining phosphorylation of HTT S13.

Similarly, they demonstrate that overexpression of TBK1 but not kinase dead TBK1 (KD) can increase S13 phosphorylation in non-neuronal (human epithelial embryonic kidney HEK293T) cell culture. The authors use overexpression of TBK1 (or TBK1 KD) and HTT to examine its specificity and efficiency for phosphorylating full-length and N-terminal fragments of HTT in HEK293T, primary neuronal culture and in C. elegans in vivo, and examine the effect of TBK1 on HTT abundance and cellular localization. They also find that an activation of autophagy by TBK1 may reduce HTT aggregation through a reduction in HTT abundance due to increased autophagic flux, suggesting that a therapeutic strategy to activate TBK1 and autophagy may be useful to treat HD.

This manuscript has many new important findings relevant to HTT S13 phosphorylation and clearance that are novel and convincing, and does suggest a new therapeutic target, TBK1, for HD drug development. I like this work very much and it is well written and engaging.

It is disturbing, however, that in several instances the claims are not appropriately discussed in the context of earlier literature. As in the abstract, the claim of "the lack of natural kinases that regulate N-terminal phosphorylation" is false, as IKKbeta was previously shown to phosphorylate HTT S13 in vitro, in cell culture, and in vivo (PMID: 20026656, PMID: 31088970). Note that reduction of IKKbeta levels in both wt or in R6/1 HD mouse striatum significantly increased levels of full-length mouse HTT and reduced levels of S13 phosphorylation on full-length mouse HTT in vivo. IKKbeta knockdown also further impaired HD behavior and increased neurodegeneration and microglial activation in R6/1 mice. These results for IKKbeta are in the same vein as the results being described in this manuscript for TBK1 involving HTT S13, but are not discussed.

Response: We thank the referee for pointing out these studies. Our references to the lack of natural kinases were related to phosphorylation at all possible phosphorylation sites within the N17 peptides. We have modified our statements to reflect this and highlighted prior discoveries related to IKKbeta when discussing phosphorylation at S13. See, page 2 line 4 and page 27 line 9-13 in the discussion section)

I also find the following statement dismissive of other relevant literature: "We recently showed that phosphorylation of HTT at S13 and/or S16 inhibits mutant HTTex1 aggregation in vitro (Deguire et al., 2018) and increases its conformational flexibility (Daldin et al., 2017). A direct comparison of the effect of phosphomimetics and bona fide phosphorylation at these sites also showed that the phosphomimetics (S13D/S16D) only partially reproduced the effect of phosphorylation on aggregation and did not reproduce the effect of single and double phosphorylation at these residues on the helical conformation of N17 (Deguire et al., 2018)."

Response: It was not our intention at all to dismiss the relevant literature. This statement reflects our findings concerning the extent to which phosphomimetics reproduce the effect of phosphorylation at S13/S16 and also T3 *in vitro* (Chiki, DeGuire et al., 2017, DeGuire et al., 2018). In these two papers, we showed that the phosphomimetics 1) only partially reproduce the effect of phosphorylation on the aggregation of mutant Httex1; 2) do not reproduce the effect of phosphorylation at T3 on increasing the helicity of N17; and 3) do not reproduce the effect of phosphorylation at S13/S16 on reversing T3 phosphorylation induced helical conformation of N17.

I do not agree that work with phosphomimetics from several groups is completely irrelevant and should be dismissed.

Response: We agree and it was not our intention to convey this message. We simply tried to reflect on the limitation of the phosphomimetic approach based on our in vitro studies and point out that because of their irreversible nature, they do not reflect the equilibrium between the phosphorylated and unphosphorylated states of the protein. We have now modified the statement and refer to these limitations only in the contexts where this direct comparison was made, i.e. the comparison of the aggregation inhibition effect of phosphomimetic and authentic phosphorylation on mutant HTTex1 (page 4 line 15-16).

We have also removed the statement "they highlight the limitations of using phosphomimetics" page 5 line 5

In fact, in 2009 S13/16D HTT peptide showed in vitro reduced aggregation (PMID: 20064390) similar to Deguire 2018, and HTT S13/16D exon 1 fragment abundance in cell culture was found to be reduced (PMID: 20026656), which would support the findings of this manuscript under review.

Response: This is true. We also showed that when both residues are mutated to D, they reduce the aggregation of mutant Httex1 in vitro. However, this is only the case when both S13 and S16 are mutated to aspartate, S13D/S16D. We showed that 1) the effect of phosphorylation at both residues results in stronger inhibitory effects compared to the phosphomimetics; 2) the phosphomimetic mutations do not reproduce the inhibitory effect of phosphorylation at the level of single residues, i.e. S13D or S16D, compared to bona fide phosphorylation at these residues (Figure 9 (DeGuire et al., 2018)).

Within their manuscript, the authors include S13/16A phosphoresistant mutation, but not S13/16D phosphomimetic mutation in their studies, inclusion of which might have informed their work with TBK1-mediated HTT phosphorylation in their system in cells.

In the context of dopaminergic neurons in adult Drosophila brain (PMID: 28934390), mimicking phosphorylation of S13 (S13D) within mutant HTT exon 1 protein increases aggregation (the opposite of what has been found in vitro by the authors and others), suggesting that in vivo phosphorylation of S13 may create an oligomerized autophagic clearance intermediate.

Response: This is difficult to assess because *in vitro* we use proteins that are homogeneously modified (100% phosphorylated) and compare their structure and aggregation properties to the corresponding unmodified proteins, whereas in *Drosophila*, the fraction of phosphorylated at S13 HTT is not known.

This may be activated by IKKbeta- or TBK1-mediated HTT phosphorylation and cleared with the induction of autophagic flux activated by both kinases - this is not considered in the present manuscript. Oligomerization has been found to guide pathway choice between proteasomal and autophagic degradation (PMID: 28504708). As the authors have examined in vitro effects of phosphorylation of N17 to reduce aggregation, they need to also consider that in vivo, TBK1 and IKKbeta may both increase the formation of oligomerized autophagic clearance intermediates through phosphorylation of HTT S13. I therefore feel that the authors need to represent earlier literature more fairly, even though all these paper were indeed cited in the text.

Response: We thank the referee for bringing this to our attention and apologize for this. We have amended the discussion to ensure an accurate representation and citation of these previous studies (page 27 line 21 - page 28 line 5).

I have some questions regarding several of the figures, please see numbered comments below:

1. *Figure 1DE and S2AB: What antibody is being used to detect HTT here? I would appreciate the authors putting the anti-HTT antibody used for each figure into the figure legend.*

Response: We used anti huntingtin antibody MAB5492, pS13 HTT (CHDI-90001039-1), anti pT3 (CHDI-90001528-1) and pS16 (generated in house) (Bustamante, Ansaloni et al., 2015, Cariulo, Azzollini et al., 2017, Cristina Cariulo, 2019). We now specify all the HTT antibodies used in the figure legend for each figure.

As far as I can tell, 1D may use anti-eGFP? HTT exon 1 protein is greatly stabilized by fusion to a C-terminal GFP tag, and the GFP tag can be post-translationally modified impacting its clearance (PMID: 15064418). Does this eGFP tag impact the clearance of 16Q and 72Q HTT exon 1 protein in their hands and what impact does that have on the levels of total HTT protein that are changing in this experiment?

Response: We used anti huntingtin antibody MAB5492.
We do not believe that the presence of the GFP tag influences the reduction in Httex1 levels induced by TBK1, because we observed similar reduction in the levels and aggregation of untagged HTTex1 72Q (Fig R3 A, B), HTTN586 92Q (Fig R3 C) and HTT-FL 23Q (Fig R3 D).
Also, in our mass spectrometry studies (MS/MS analysis), we did not observe increased post translational modification of eGFP in our when TBK1 and HTTex1 eGFP were co-expressed (See, Fig R4).

Although Origene's Myc-tagged IKKbeta and TBK1 constructs appear to be equivalently expressed by Western with anti-Myc, this does not mean that they are equally active with Myc tag fusion.
Also, IKKalpha, IKKbeta and IKKgamma often work together within the IKK complex, while TBK1 is simply a dimer of identical subunits – the authors don't have all 3 IKK subunits in their in vitro or cell culture systems, which might impact the relative data intensity when comparing IKKbeta to TBK1 activity (Figures 1DE and S2AB). Note it has previously been published that both IKKalpha and IKKbeta catalytic subunits can directly phosphorylate HTT S13 on wt and mutant HTT exon 1 protein in vitro, and overexpression of IKKalpha in cell culture also increases phosphorylation of HTT S13 and S16 (PMID: 20026656). In addition, the IKK complex regulatory subunit IKKgamma has been shown to directly interact with HTT's proline-rich region adjacent to N17 (PMID: 15371500) - the lack of the regulatory subunit in the in vitro kinase assay may slow recruitment as in S2B. Without the entire IKK complex in the authors' studies, I believe it is impossible to suggest that TBK1 is a stronger S13 kinase than IKKbeta functioning within context of the IKK complex, which is present in vivo. What the authors can say is that they both activate phosphorylation of HTT S13 in their hands.

Response: We agree with the referee that comparing IKK-beta and TBK1 directly may not be appropriate.
We have modified the text to make it clear that "both IKK-beta and TBK1 activate phosphorylation of HTT S13 in our hands and added a sentence to highlight the point made by the referee as follows "However, this comparison may not be accurate as the activity of IKKβ could be influenced by IKKα alpha and IKKγ, which may work together within the IKK complex". (page 8 line 8-10).

2. *Figure 2: 2A: Is this a Western using anti-eGFP antibody to detect HTT?*

Response: We used anti huntingtin antibody MAB5492, pS13 HTT (CHDI-90001039-1), anti pT3 (CHDI-90001528-1). This is now clearly specified in the figure legend.

Note that there is residual pS13 activity in the wt and the T3A 16Q with TBK1 KD overexpression - there is another active kinase in the HEK293T cells - how about IKK complex?

Response: We did not probe whether IKK-complex regulates the HTT S13 phosphorylation in HEK293 cells as it was not the focus of this project. We agree that this is a possibility that is worth testing in future studies. We have added a sentence, ". Interestingly, we observed a residual phosphorylation at S13 in TBK1 KD, which could be to other endogenous kinases, including but not limited to IKKβ" (page 9 line 8).
We also mention the possible regulation of endogenous S13 phosphorylation by other kinases in the page 10 line 19-23.

2C: What antibody is being used for anti-HTT?

Response: We used anti huntingtin antibody MAB2166, pS13 HTT (CHDI-90001039-1). This is now clearly specified in the figure legend.

Quantitation by Singulex assay from the authors' previous 2020 paper (PMID: 31677786) would suggest MW1/2B7 was used in the Singulex assay? 2B7 is a monoclonal antibody with a small epitope including HTT lysine K9, which becomes acetylated (potentially destroying the 2B7 epitope) with S13 phosphorylation (PMID: 20026656). Therefore 2B7 may not detect the modified species cleared by autophagy - it may not be a total HTT antibody and should not be used to quantitate relative levels of pS13.

Response: We thank the referee for pointing this out. We used MW1/2B7, as described in materials and methods and in our previous publication (Cristina Cariulo, 2019).
We believe that using 2B7 does not have much impact on the detection of HTT in these assays.

a. We did not observe K9 acetylation in our mass spectrometry analysis of 2 samples of HTTex1 and 4 samples of HTT-FL purified from HEK293 cells and Htt-FL isolated from mouse brain homogenate (See Fig R5). Thus, we do not believe that this modification is present in significant amounts to interfere with the assay.
b. We have also demonstrated previously that total levels of HTT measured in mouse brains by MW1/2B7 do not differ from those measured using MW1/2166 (Fig 4 B and C) or MW1/ HDB4ED10 (Fig S5B)(Cariulo et al., 2017).

I note that TBK1 overexpression in the context of HTT N548 55Q does not reduce the fragment - is this because the authors are detecting with 2B7?

Response: As mentioned now in the figure legend, we used MAB2166 antibody.
This may be due to time of co-expression was only 24h instead of the 48h used for HTT exon 1. We observed the reduced levels of HTTN586 92Q and HTT-FL 23Q upon co expression of TBK1 at late timepoints (48 hours to 96 hours) (Fig R3 C and D). It is also possible that longer fragments may exhibit different stability and behave differently.

Note good levels of p13 phosphorylation again without TBK1 overexpression - is this IKK? 2EF: What antibody is being used for anti-HTT - 2B7?

Response: We thank the referee for pointing this out. We have not probed whether this could be particularly due to IKK complex in this project, but we cannot rule this out. As mentioned in page 10 line 19 and 23, other kinases may be responsible for this residual phosphorylation.
We used anti huntingtin antibody MAB2166, pS13 HTT (CHDI-90001039-1).

The legend says the authors are comparing full-length 23Q vs. 48Q HTT, but the figure shows S13/16A? Are these wt 48Q and S13/16A 48Q, without 23Q in the figure?

Response: We thank the referee for pointing out this mistake. We apologize, it is HTT 48Q wt and S13/16A, we have corrected the legend.

Why are levels of TBK1 KD so low in this experiment? - it is not a control for wt TBK1 in this panel.

Response: We agree with the referee that the TBK1 KD expression levels are low. In some of our experiments TBK1 KD expression is less efficient but definitely detectible. These observations are similar to what has been reported in previous studies (Fig.1 A, B (Nakhaei, Sun et al., 2012), Fig 1 A (Harris, Oliere et al., 2006)). Nonetheless, coexpression of TBK1 KD does not increase phosphorylation of HTT by even a small order above the residual pS13 levels across the experiments presented in Fig 1 D, Fig.2 A, C, E, G.

Note that if the authors have high levels of pS13 in the wt 48Q this might reflect upregulation of IKK activity with polyQ expanded HTT expressed consistent with the work of Ali Khoshnan/Paul Patterson from 2004 PMID: 15371500.

Response: We thank the referee for pointing this out. We did not prob whether this could be particularly due to upregulation of IKK complex in the context of this study. The proposed IKK upregulation upon polyQ expanded HTT expression does not seem to correlate with the observed residual S13 phosphorylation because:

a) Our previous data from Q111 HD model show that mHtt has less residual S13 phosphorylation [Fig 4 (Cristina Cariulo, 2019)].
b) The residual S13 phosphorylation is reduced slightly on HTTex1 72Q compared to HTTex1 16Q (Fig 1 D, Fig 2A)

In the text, we highlighted the possibility that other kinases could be responsible for residual pS13 levels, see page 10 line 19 and 23.

Once again the Singulex assay 2F does not appear to be validating the Western in 2E.

Response: We agree with the referee that the western blot data and Singulex does not match. This could be possibly because

a) Western blotting is just not as sensitive as the Singulex assay.
b) In western blotting, we are detecting and quantifying only the intact HTT, whereas the much sensitive Singulex assay from cell lysates can capture fragments or low. Mol. wt. HTT species.

2G: What antibody is being used for anti-HTT detection - I see that D7F7 was used for the IP of endogenous wt HTT in rat primary striatal neurons, and here authors are observing reduced levels of full-length HTT and increased HTT phosphorylation with TBK1 overexpression. This figure is nice, but the antibodies used need to be written in the legend.

Response: We used anti huntingtin antibody MAB2166 and pS13 HTT (CHDI-90001039-1). This is now clearly specified in the figure legend.

Is LV GFP expression increasing levels of full length wt HTT over LV TBK1 KD, suggesting a blockage of HTT autophagic clearance by GFP?

Response: HTT levels in LV GFP and TBK1 KD were not significantly different across experiments and hence we don't believe that GFP expression influence HTT degradation.

3. *Figure 3: Is the antibody detecting the HTT construct on the Western anti-eGFP?*

Response: We used anti huntingtin antibody MAB5492, pS13 HTT (CHDI-90001039-1), anti pT3 (CHDI-90001528-1). This is now clearly specified in the figure legend.

3A: Again, what impact does eGFP have on abundance of these exon 1 proteins (as eGFP fusion itself stabilizes HTT exon 1 protein), and

Response: We thank the referee for highlighting this point. As mentioned above, the GFP tag does not influence TBK1-induces changes in the levels of total HTT (Fig R3, Fig R4).

can IKKbeta and TBK1 directly be compared based on overexpression of myc-tagged constructs without co-overexpression of IKKalpha and IKKgamma with IKKbeta?

Response: We agree with referee's concern regarding the TBK1 and IKK- beta comparison. Despite the fact that IKK-Beta subunit alone phosphorylates unexpanded HTTex1 (Fig 1 D (Thompson, Aiken et al., 2009)), we agree that comparing the efficiency may not be reasonable without co-expression of IKKalpha and IKKgamma with IKKbeta.
We have modified the text to highlight this point and no longer emphasize the differences in phosphorylation efficiency of IKKbeta alone and TBK1(page 24 line 21- page 25 line 1).

It does look like IKKbeta and TBK1 are reducing HTT levels both in the whole cell extract and in aggregates independent of phosphorylation of S13/16.

Response: We agree.

Might S13/16D have done the same without the kinases - is expression of the kinase essential here for increased clearance and reduced aggregates - what is the contribution of phosphorylation alone to abundance and aggregation? I think the authors have done themselves a disservice by not using S13/16D mutants for figure 3, although technically the data is solid.

Response: HTT S13/S16D does not influence soluble HTT levels but did reduce mutant HTT aggregation.

a) In fact, studies by other groups showed similar levels of soluble HTTex1 97Q S13/16D, or S13D compared to HTTex1 97Q (Thompson et al., 2009) (Branco-Santos, Herrera et al., 2017) (fig2a and fig.1D respectively), although reduced levels of HTTex1 25Q but not HTTex1 46Q S13/16E has been reported in one study (Thompson et al., 2009) (Fig 3C)

In our studies we showed that phosphorylation at S13 is a major contributor to inhibiting the aggregation of mutant HTT (Fig.7 A, B). Under conditions where TBK1-mediated autophagic clearance, but not phosphorylation of mutant HTT is disrupted (TBK1 delta 690-713), the aggregation inhibitory effects of TBK1 is strongly dependent on phosphorylation at S13 (Fig.7 A, B).

Therefore, HTT S13 phosphorylation may increase the soluble pool of HTT and contribute to enhanced clearance of HTT (Maharjan et, al unpublished).

Figure S4B, note that 72Q with TBK1KD has more nuclear localization than 16Q with TBK1KD - is this significant and does this reflect endogenous activation of IKK with mutant HTT expression as previously described (PMID: 15371500)?

Response: The nucellar localization of 72Q vs 16Q with TBK1 KD expression shows a trend of increase but is not significant.
We have not probed for any activation of IKK under these conditions within the scope of this project.

4. *Figure 5: Is the anti-HTT antibody anti-YFP in these experiments?*

Response: We used anti huntingtin antibody MAB5492, pS13 HTT (CHDI-90001039-1), anti pT3 (CHDI-90001528-1). This is now clearly specified in the figure legend.

Isn't Unc-51 generally considered an Atg1/ULK2/ULK1 homolog, not a TBK1 homolog? The IKK complex and TBK1 are not conserved in C. elegans, and there is no "HTT" - N17 is not present or conserved.

Response: unc-51 and ikke-1 come up as most probable hits using the blast, also UCSD signaling gateway ortholog data mentions unc-51 and ikke-1 as top orthologues (http://www.signaling-gateway.org/molecule/ query?afcsid=A001597&type=orthologs &adv=latest).

TBK1 is not greatly impacting levels of transiently transfected soluble N513 fragment but does reduce aggregates - does YFP fusion stabilize N513 in C. elegans? Is TBK1 in this case actually clearing aggregates, and not soluble oligomers?

Response: We do not know whether YFP stabilizes HTT N513 in C. elegans.
We did not probe whether TBK1 expression clears already formed HTT aggregates in *C. elegans*. TBK1 expression here was constitutive and it could not be induced after the formation of Htt aggregates and therefore we cannot comment on the effect of TBK1 on the clearance of already formed aggregates in this model.
Based on our cellular data (Fig 3 H, I), HTT clearance happens before aggregation occurs resulting in a reduced number of HTT aggregates. In addition, our in vitro phosphorylation studies show that TBK1 does not phosphorylate or disassemble recombinant preformed fibrils.

We have not assessed the effect of TBK1 phosphorylation on HTT oligomers because these species are very difficult to capture or populate reproducibly in cellular and animal models and *in vitro* as we indicated in the page 27 line 23-page 28 line 5. We are actively working to develop methods to stabilize and isolate oligomers.

pS13 does not appear unstable in this system - is the autophagic mechanism for human N513 not precisely conserved in C. elegans?

Response: We hypothesize that at least in part autophagic mechanism seem to be conserved in *C. elegans*. Supporting this view, a recent report shows that in worms ikke-1 (TBK1 orthologue) acts on allo-1 (autophagy receptor in worms like p62 and OPTN) which then binds to ubiquitinated substrates (Sato, Sato et al., 2018). It is likely that TBK1 may regulate allo-1 and hence activate autophagy in worms.

TBK1 is highly related to IKK-epsilon (49% identical), so it does make sense that C. elegans IKK-epsilon is the endogenous kinase available to phosphorylate human HTT S13 in this system, but it does not impact stability of soluble or insoluble human N513. Is endogenous C. elegans IKK-epsilon responsible for the basal pS13 activity in 5D?

Response: Yes, our data in Fig 5 H show endogenous C. elegans ikke-1 (IKK-epsilon) is responsible for the basal pS13 activity so it may be also the case for the samples in 5D.

Does human IKK-epsilon regulate HTT pS13 phosphorylation in HEK293T cells as well? Based on homology I suspect it does. Is it a relevant pS13 kinase in vivo like IKKbeta, as TBK1 may not be based on figure S3I?

Response: We have not probed whether IKK- epsilon regulates the HTT S13 phosphorylation as our current focus was TBK1. We will test this hypothesis in future studies.

Was it one of the 300 kinases tested in the in vitro kinase original screen?

Response: Yes, it was tested and it did not show any phosphorylation activity on HTTex1.

5. *Figure 6 and S6: TBK1 is well characterized to activate autophagy, and it is also well defined that HTT aggregate numbers are reduced with activation of autophagy, although it is good to see in the authors' system.*

Response: We agree that reduction of mutant huntingtin aggregates by activating autophagy using various agents and approaches is well established in a system identical to our system.

a) Treatment of HTTex1 74Q GFP expressing HEK293 cells with autophagy inducer "genistein" resulted in significant reduction of aggregates and soluble HTT (Pierzynowska, Gaffke et al., 2018)
b) Treatment of HTTex1 Q74 GFP expressing COS-7 cells with autophagy inducer "rapamycin" resulted in reduction of aggregates and soluble HTT levels (Ravikumar, Duden et al., 2002).

Treatment of HTTN171-82Q expressing mouse model with autophagy inducer "Rilmenidine" resulted in reduction in soluble HTT levels (Rose, Menzies et al., 2010).

6. *Figure 7: Figure 7B bottom, and 7D label is incorrect - the delta 690-713 is not written in so it is confusing.*

Response: Our apologies, we thank the referee for pointing out. Now we corrected this error.

7. *Figure S3: A panel from S3A with mass spec data is occluding figure S3CDFG - a formatting issue with the figure.*

Response: Our apologies for this formatting error. We have corrected the formatting.

What antibody is used for S3E.

Response: We used anti huntingtin antibody D7F7 and pS13 HTT (CHDI-90001039-1) This is now specified in the figure legend.

(I am assuming anti-eGFP is used for S3CD)?

Response: Yes, we have used anti GFP antibody for IP and for detection we used MAB5492 for HTT and anti MYC of TBK1. We have indicated the same in the legend.

Figure S3GH has controls on the end and everything else seems higher, even with titration of drugs. I find this figure difficult to believe.

Response: As the referee points out all drug treatments show higher levels of HTT and just show the noted trend of increase in HTT. The titration of drug was not done with extensive doses because

a) Number of primary cells needed for each of the treatments was limiting and endogenous Htt expression is very low in the primary culture system.
b) Variation between the different experiments were high.

What total anti-HTT antibody is used for S3IJ?

Response: We used anti huntingtin antibody D7F7 and anti pS13 HTT (CHDI-90001039-1). This is now clearly specified in the figure legend.

For S3EF and IJ, what is the ratio of pS13/total HTT? I can see that full-length HTT accumulates with TBK1 knockdown, but more pS13 reactivity is present as well. Does the ratio of pS13/total HTT drop with TBK1 reduction?

Response: The ratio of pS13HTT/total HTT does not show any significant change in these experiments.

8. *Page 1 of manuscript: I do not believe the manuscript shows that "TBK1-mediated neuroprotective effects are due to phosphorylation-dependent inhibition of mutant HTTex1 aggregation" - it shows that HTT abundance is reduced with pS13, and that autophagy is activated thereby reducing abundance (which both*

may result in reduced aggregates, but not a direct inhibition of aggregation within N17, so perhaps the way this is written is misleading)

Response: Our apologies for not being clear. Our aim here was to emphasize that the combination of both phosphorylation at S13 and TBK1 mediated autophagic clearance of HTT are responsible for neuroprotective effects.
The effects on HTT levels and aggregate formation become strongly dependent on the S13 phosphorylation state upon disruption of TBK1 interactions (TBK1 delta 690-713) with autophagy adaptor proteins.
We observed significant reduction of aggregate formation by HTT 72Q, but not the HTT 72Q S13A/S16A mutant, upon coexpression of these proteins with TBK1 delta 690-713, which does not promote autophagic clearance of mutant HTT (Fig.7 A, B). This shows that pS13 phosphorylation is sufficient to significantly reduce the aggregation of mutant HTTex1

9. *Page 5: Ochaba et al 2019 recently showed that IKKbeta increased HTT S13 phosphorylation in HD and wt control mice in vivo.*

Response: Our apologies for not being clear. We have modified the sentence as follows,
"Previously, IKKβ overexpression was shown to phosphorylate HTT at these residues (Thompson, Aiken et al., 2009) and at residue T3 (Bustamante, Ansaloni et al., 2015), and to increase HTT S13 phosphorylation in HD and wildtype mice (Ochaba, Fote et al., 2019)".

10. *Page 11, line 5, Marc Diamond's paper should be included in the citations here PMID: 23319588.*

Response: We thank the referee for pointing this out. We have now cited this work of Marc Diamond's, see page 11 line 11.

11. *Page 12, line 2, Saudou's 2007 paper does not deal with S13/S16 - it only deals with S1181/S1201 which are cdk5 sites. Gu et al, 2009 PMID: 20064390 is the missing reference for here.*

Response: We thank the referee for pointing out this mistake. We have added Gu et al, 2009 and removed Saudou et al, 2007-page 12 line 3 and 5.

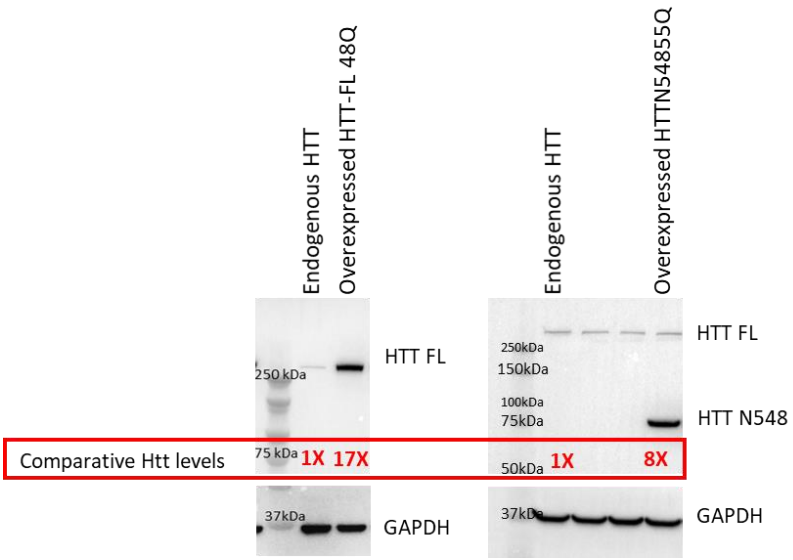

Figure R1: Comparative HTT overexpression level:
Representative western blot showing the endogenous HTT and overexpressed HTT-FL and N548 (ab-MAB2166) from the lysates of HEK293 cells transfected with plasmids pCDNA empty vector (EV), HTT-FL and N548 for 24 hours. Red box indicates the comparative expression level.

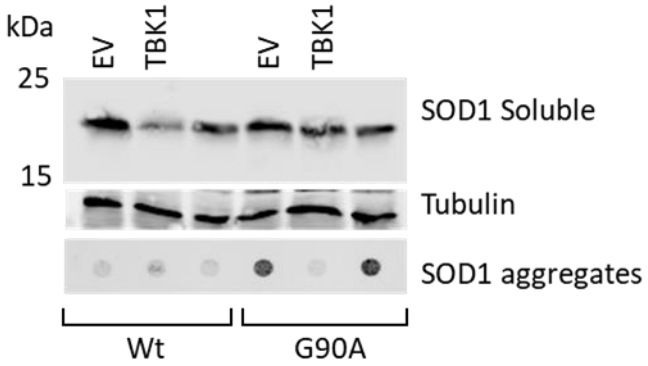

Figure R2: TBK1 expression reduces SOD1 level:
Representative western blot showing the soluble and insoluble fractions from the lysates of HEK293 cells after 48 hours of coexpression of TBK1 or EV with SOD1 wt or SOD1 90A.

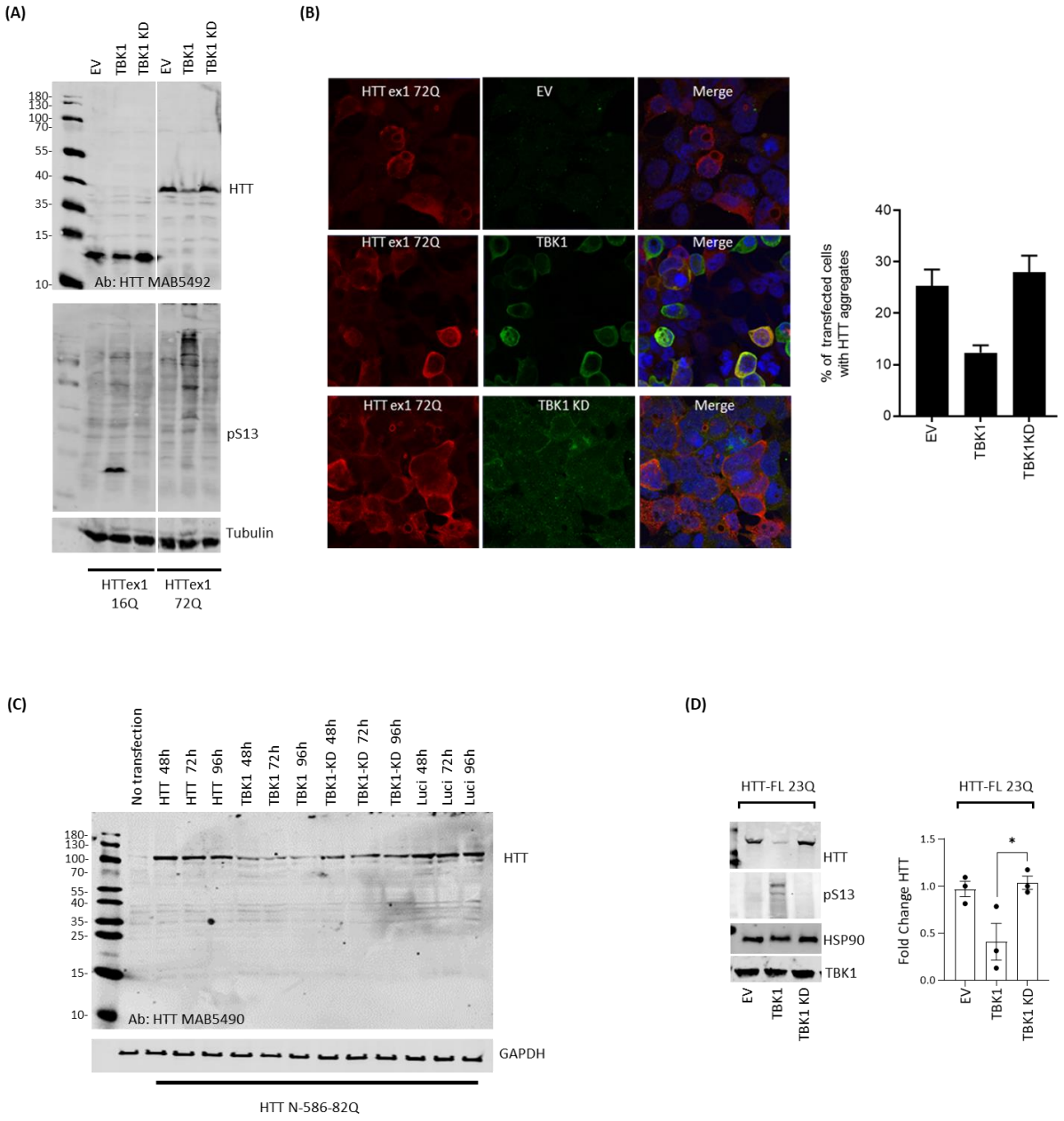

Figure R3: TBK1 reduces the levels of untagged HTT:
(A) Western blot of HTT (detected by ab-MAB5492) and Htt pS13 (detected by ab-CHDI-90001039-1) from HEK293 cell lysates co-transfected with HTTex1 16Q or 72Q with TBK1 or TBK1 KD for 48 hours.
(B) Immunofluorescence image of HTT 72Q (detected by ab-MAB5492) from HEK293 cells after 48 hours of co-transfection of HTTex1 72Q together with EV or TBK1 or TBK1 KD, with graph indicating the percentage of cells showing HTT aggregates (graph represent mean ± SEM form duplicate slides).
(C) Representative western blot of HTT N586 82Q (detected by ab-MAB5490) from HEK293 cell lysates co-transfected with HTTN586 92Q together with TBK1 or TBK1 KD for 48,72 and 96 hours.
(D) Representative western blot of overexpressed HTT-FL (detected by ab-MAB5490) and HTT pS13 (detected by ab-CHDI-90001039-1) from HEK293 cell lysates co-transfected with HTT-FL 23Q together with TBK1 or TBK1 KD for 72 hours with the quantification of fold change HTT compared to TBK1 KD (normalized to housekeeping protein) on the right. Graph report mean ± SEM.

Significance was assessed using one-way ANOVA, < 0.001 - ***, 0.001 to 0.01 **, 0.01 to 0.05 *, ≥ 0.05 ns.

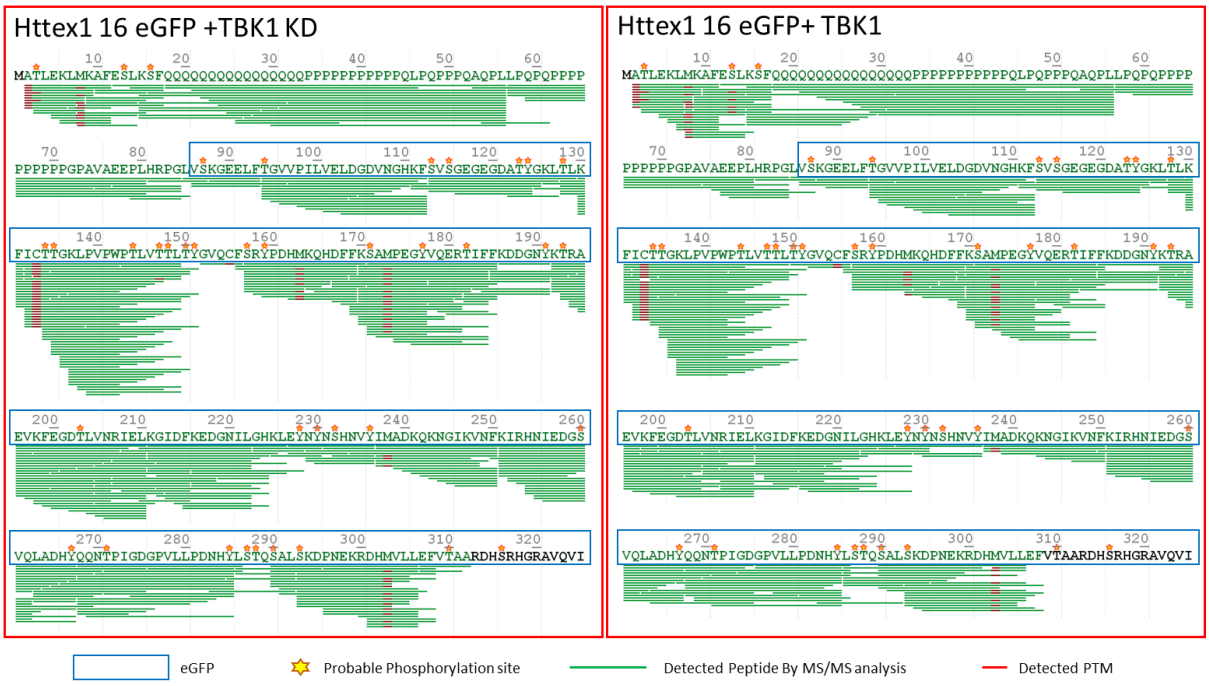

Figure R4: TBK1 does not change the phosphorylation state of the eGFP tag:
HTTex1 16 eGFP immunoprecipitated using eGFP antibody from HEK293 cell lysates after coexpression of HTTex1 16Q eGFP with TBK1 or TBK1 KD for 48 hours were loaded to SDS-PAGE and bands corresponding to HTTex1 eGFP were subjected to in-gel digestion using chymotrypsin and LC MS/MS measurement were performed (see methods). HTT peptide with S13 phosphorylation were detected upon TBK1 expression but eGPF peptides with phosphorylation at any probable sites (marked above by star) were not detected.

(A)

HTTex1 16Q eGFP+ TBK1

PID	Prot. Rank	Pos.	Sequence	ilycan	PEP 2D	Mods (variable)
46 |...	1	2	M.[+42.01056]ATLEKLM		4e-06	NTerm(Acetyl / 42.0106)
47 |...	1	2	M.ATLEKLM		0.01	
48 |...	1	3	A.TLEKLM.K		0.89	
49 |...	1	3	A.TLEKLM		0.79	
50 |...	1	5	L.EKLM[+15.99492]KAFESL.K		4.6e...	M4(Oxidation / 15.9949)
51 |...	1	5	L.EKLM[+15.99492]KAFESL.K		1.1e...	M4(Oxidation / 15.9949)
52 |...	1	5	L.EKLM[+15.99492]KAF.E		2.9e...	M4(Oxidation / 15.9949)
53 |...	1	5	L.EKLM[+15.99492]KAF.E		2.4e...	M4(Oxidation / 15.9949)
54 |...	1	6	E.KLM[+15.99492]KAF.E		1.1e...	M3(Oxidation / 15.9949)
55 |...	1	6	E.KLMKAF.E		0.0002	
56 |...	1	7	K.LM[+15.99492]KAF.E		0.74	M2(Oxidation / 15.9949)
57 |...	1	7	K.LM[+15.99492]KAF.E		0.98	M2(Oxidation / 15.9949)
58 |...	1	7	K.LMKAFESL.K		0.39	
59 |...	1	7	K.LMKAF.E		0.16	
60 |...	1	8	L.MKAFES[+79.96633]LKSF.Q		3.2e...	S6(Phospho / 79.9663)
61 |...	1	8	L.MKAFES[+79.96633]LKSF.Q		0.00...	S6(Phospho / 79.9663)
62 |...	1	8	L.M[+15.99492]KAFES[+79.96633]LKSF...		9e-06	M1(Oxidation / 15.9949); S6(Phospho / 79.9663)
63 |...	1	8	L.M[+15.99492]KAFESLKSF.Q		5.8e...	M1(Oxidation / 15.9949)
64 |...	1	8	L.M[+15.99492]KAFESLKSF.Q		0.00...	M1(Oxidation / 15.9949)
65 |...	1	8	L.M[+15.99492]KAFESLK.S		5.7e...	M1(Oxidation / 15.9949)
66 |...	1	8	L.M[+15.99492]KAFESL.K		0.011	M1(Oxidation / 15.9949)
67 |...	1	8	L.M[+15.99492]KAFESL.K		0.19	M1(Oxidation / 15.9949)
68 |...	1	8	L.MKAFESL.K		0.0079	
69 |...	1	9	M.KAFES[+79.96633]LKSF.Q		1.8e...	S5(Phospho / 79.9663)
70 |...	1	9	M.KAFES[+79.96633]LKSF.Q		0.53	S5(Phospho / 79.9663)

HTTex1 16Q eGFP+TBK1 KD

PID	Prot. Rank	Pos.	Sequence	ilycan	PEP 2D	Mods (variable)
49 |...	1	2	M.[+42.01056]ATLEKLM		2.1e...	NTerm(Acetyl / 42.0106)
50 |...	1	2	M.[+42.01056]ATLEKLM		0.00...	NTerm(Acetyl / 42.0106)
51 |...	1	2	M.[+42.01056]ATLEKL		0.1	NTerm(Acetyl / 42.0106)
52 |...	1	3	A.TLEKLM.K		0.78	
53 |...	1	3	A.TLEKLM		0.049	
54 |...	1	3	A.TLEKLM		0.11	
55 |...	1	5	L.EKLM[+15.99492]KAFESL.K		6.9e...	M4(Oxidation / 15.9949)
56 |...	1	5	L.EKLM[+15.99492]KAFESL.K		4.2e...	M4(Oxidation / 15.9949)
57 |...	1	5	L.EKLM[+15.99492]KAFESL.K		0.4	M4(Oxidation / 15.9949)
58 |...	1	5	L.EKLM[+15.99492]KAF.E		0.0013	M4(Oxidation / 15.9949)
59 |...	1	6	E.KLM[+15.99492]KAF.E		0.0084	M3(Oxidation / 15.9949)
60 |...	1	6	E.KLMKAF.E		0.011	
61 |...	1	7	K.LM[+15.99492]KAF.E		0.59	M2(Oxidation / 15.9949)
62 |...	1	8	L.M[+15.99492]KAFESLKSF.Q		5.6e...	M1(Oxidation / 15.9949)
63 |...	1	8	L.M[+15.99492]KAFESLK.S		0.0011	M1(Oxidation / 15.9949)
64 |...	1	8	L.M[+15.99492]KAFESL.K		0.013	M1(Oxidation / 15.9949)

B)

HTT-FL 23Q

PID	Prot. Rank	Pos.	Sequence	ilycan	PEP 2D	Mods (variable)
1 | ...	1	1	-.MATLEKLM[+15.99492]K[+114.042...		0.98	M8(Oxidation / 15.9949); K9(GlyGly / 114.0429)
2 | ...	1	2	M.[+42.01056]ATLEKLM[+15.99492]...		4.3e...	NTerm(Acetyl / 42.0106); M7(Oxidation / 15.9949)
3 | ...	1	2	M.[+42.01056]ATLEKLM[+15.99492]...		1.3e...	NTerm(Acetyl / 42.0106); M7(Oxidation / 15.9949)
4 | ...	1	2	M.[+42.01056]ATLEKLMKAF.E		2.7e...	NTerm(Acetyl / 42.0106)
5 | ...	1	2	M.AT[+79.96633]LEK[+114.04293]L.M		0.29	T2(Phospho / 79.9663); K5(GlyGly / 114.0429)
6 | ...	1	9	M.K[+114.04293]AFESL.K		0.28	K1(GlyGly / 114.0429)
7 | ...	1	9	M.K[+114.04293]AFESL.K		0.8	K1(GlyGly / 114.0429)
8 | ...	1	10	K.AFESLK[+114.04293]S.F		0.92	K6(GlyGly / 114.0429)
9 | ...	1	12	F.ESLK[+114.04293]SF.Q		0.18	K4(GlyGly / 114.0429)
10 |...	1	18	F.QQQQQQQQQQQQQQQ.Q		1	

HTT-FL 48Q

PID	Prot. Rank	Pos.	Sequence	ilycan	PEP 2D	Mods (variable)
1 | ...	1	2	M.[+42.01056]AT[+79.96633]LEKLM...		9.3e...	NTerm(Acetyl / 42.0106); T2(Phospho / 79.9663)
2 | ...	1	2	M.[+42.01056]ATLEKLM[+15.99492]...		5.5e...	NTerm(Acetyl / 42.0106); M7(Oxidation / 15.9949)
3 | ...	1	2	M.[+42.01056]ATLEKLM[+15.99492]...		4.6e...	NTerm(Acetyl / 42.0106); M7(Oxidation / 15.9949)
4 | ...	1	2	M.[+42.01056]ATLEKLMKAF.E		4.2e...	NTerm(Acetyl / 42.0106)
5 | ...	1	2	M.[+42.01056]ATLEKLM.K		1.6e...	NTerm(Acetyl / 42.0106)
6 | ...	1	3	A.TLEKLM[+15.99492]K.A		0.97	M6(Oxidation / 15.9949)
7 | ...	1	5	L.EK[+114.04293]LMKAFES.L		0.98	K2(GlyGly / 114.0429)
8 | ...	1	7	K.LM[+15.99492]K[+114.04293]AFE...		0.92	M2(Oxidation / 15.9949); K3(GlyGly / 114.0429)
9 | ...	1	8	L.M[+15.99492]K[+114.04293]AFESL.K		0.83	M1(Oxidation / 15.9949); K2(GlyGly / 114.0429)

(C)

Htt (endogenous) from mouse brain

PID	Prot. Rank	Pos.	Sequence	ilycan	PEP 2D	Mods (variable)
1 | ...	1	2	M.[+42.01056]ATLEKLM[+15.99492]...		2.8e...	NTerm(Acetyl / 42.0106); M7(Oxidation / 15.9949)
2 | ...	1	2	M.[+42.01056]ATLEKLM[+15.99492]...		1.8e...	NTerm(Acetyl / 42.0106); M7(Oxidation / 15.9949)
3 | ...	1	2	M.[+42.01056]ATLEKLMKAF.E		0.0004	NTerm(Acetyl / 42.0106)
4 | ...	1	2	M.[+42.01056]ATLEKLM[+15.99492].K		5.5e...	NTerm(Acetyl / 42.0106); M7(Oxidation / 15.9949)
5 | ...	1	2	M.[+42.01056]ATLEKLM[+15.99492].K		0.004	NTerm(Acetyl / 42.0106); M7(Oxidation / 15.9949)
6 | ...	1	2	M.[+42.01056]ATLEKLM[+15.99492].K		0.017	NTerm(Acetyl / 42.0106); M7(Oxidation / 15.9949)
7 | ...	1	2	M.[+42.01056]ATLEKLM.K		9.1e...	NTerm(Acetyl / 42.0106)
8 | ...	1	2	M.[+42.01056]ATLEKLM.K		1.3e...	NTerm(Acetyl / 42.0106)
9 | ...	1	2	M.[+42.01056]ATLEKLM.K		0.033	NTerm(Acetyl / 42.0106)
10 |...	1	2	M.[+42.01056]ATLEKLM.K		0.081	NTerm(Acetyl / 42.0106)
11 |...	1	2	M.[+42.01056]ATLEKLM		2.4e...	NTerm(Acetyl / 42.0106)
12 |...	1	5	L.EKLM[+15.99492]KAF.E		0.00...	M4(Oxidation / 15.9949)
13 |...	1	5	L.EKLM[+15.99492]KAF.E		0.29	M4(Oxidation / 15.9949)
14 |...	1	8	L.M[+15.99492]KAFESL.K		0.0049	M1(Oxidation / 15.9949)

(D)

Sample	Sample Size	Digestion	K9 Acetylation
HTTex1 16Q	2	Chymotrypsin	-
HTTex1 16Q+TBK1	2	Chymotrypsin	-
HTT-FL 23Q	2	Chymotrypsin	-
HTT-FL 48Q	2	Chymotrypsin	-
Htt-FL from mouse brain	1	Chymotrypsin	-

Figure R5: HTT does not show K9 acetylation:

(A-C) Identified peptides of HTT containing K9 residue from mass spectrometry analysis of indicated HTT samples.

(A) HTTex1 16 eGFP immunoprecipitated from HEK293 cell lysates coexpressing HTTex1 16Q eGFP with TBK1 or TBK1 KD for 48 hours. Immunoprecipitated HTTex1 was loaded to SDS-PAGE and bands corresponding to HTTex1 eGFP were subjected to in-gel digestion using chymotrypsin and LCMS measurement were performed.

(B) HTT-FL purified from HEK293 cell expression system and 1ug of protein was subjected to chymotrypsin digestion and LCMS measurement were performed.

(C) Htt was immunoprecipitated from 6mg of total mouse brain homogenate and immunoprecipitated Htt was subjected to in-gel digestion using chymotrypsin and LCMS measurement were performed.

(D) Summary table of mass spectrometry analyzed sample number and status of K9 acetylation (+ present, - absent)

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1st Revision - Editorial Decision 27th May 2020

Dear Hilal,

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been reviewed by referee #3 and the comments are provided below.

As you can see the referee appreciates the introduced changes and support publication here pending some minor changes. Can we discuss the issue raised regarding the antibodies?

When you submit your revised version will you also take care of the following points

- You have 9 keywords, but can only have 5

- The EV figure callouts are missing the word 'Figure'

- You have 7 EV figures but can only have 5. You can add two to the appendix or combine several of the EV figures. Please add the figure legends of the EV figures to the main MS file

- I see that the appendix contains results, M&M and discussions but that should really be placed in the main MS file. OK to have some M&M in appendix but most should be in main file.

- Could you deposit the MS data in database and provide the accession number in data availability section.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. I see that you have provided one can you double check the size is 550 wide by 400 high (pixels).

- I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

That should be all

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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http://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
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- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
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Referee #3:

I thank the authors for their careful rebuttal. My concerns have largely been met.

Here are my comments regarding the revised paper and the rebuttal.

General summary and opinion about the principal significance of the study, its questions and findings:

This EMBO Journal manuscript under review builds on previously published data involving the phosphorylation and autophagic degradation of the Huntingtin (HTT) protein, mutation of which causes Huntington's disease (HD). Thompson et al., 2009 first showed that 2 subunits of the IKK kinase complex, IKKalpha and IKKbeta, directly phosphorylate HTT serine (S) 13 reducing HTT stability and increasing HTT lysosomal clearance in cells. This work was continued in mice (Ochaba

et al., 2019) to show that in vivo, IKKbeta both phosphorylates HTT S13 and activates autophagy to slow neurodegeneration and HD progression. The manuscript under review beautifully extends this previously published work by showing that similar to IKKbeta, its homologous IKK family member TBK1, a kinase very important in the regulation of autophagy, can also phosphorylate HTT S13 associated with reduced HTT abundance and independent of HTT phosphorylation can also activate its clearance by autophagy leading to reduced mutant HTT aggregate formation. This comprehensive and important manuscript strongly supports the future investigation of TBK1 as a therapeutic target for the treatment of HD, as it demonstrates that TBK1 suppresses mutant HTT-induced toxicity and pathology in HD models. As the HTT protein has been shown to be a scaffold for selective autophagy (Ochaba et al., 2014, Rui et al. 2015), a process in which it may be cleared by the lysosome, the manuscript under review is an exciting update to the autophagy field as well as the HD field, and may reflect a mechanism for upregulation of HTT's autophagic scaffold function in parallel with other TBK1 substrates including HTT-interacting proteins Optineurin and SQSTM1/p62, two autophagic receptor proteins both activated by TBK1-dependent phosphorylation.

Minor concerns that should be addressed:
Page 3, line 23 and page 4, line 3 should include Thompson et al., 2009.
Page 8, line 9 - two alphas are redundant - delete the word alpha and keep the Greek letter.
Page 8, line 18 - phosphorylated is spelled incorrectly.
Page 10, line 22 - phosphorylation is spelled incorrectly.

Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion):
I have no major concerns and believe the manuscript could be published "as is". That being said, I encourage but do not require the authors to revise figure 2C-H, figure EV3G-H, figure EV4H, and figure EV6A using different total HTT antibodies. I believe the data in these figures is reproducible and valid, but may be misleading. While these are commonly used anti-HTT monoclonal antibodies, 2B7 and MAB2166 should not be used to quantitate total HTT levels as their epitopes contain lysines that become modified by acetylation (K9 and K444 respectively) that are found on phosphorylated HTT autophagic clearance intermediates relevant to this manuscript (Thompson et al., 2009, Jeong et al., 2009 PMID: 19345187 and Cong et al., 2011 PMID: 21685499). Acetylation may block the epitopes of these monoclonal antibodies so they should not be used to follow HTT clearance. Thompson et al., 2009 shows acetylation of lysine 9 together with phospho-S13 by mass spectrometry in figure 2C and demonstrates that MAB2166 does not recognize the pS13 species being cleared by autophagy shown in figure S4A (reprobed figure 3D which contains an actin control). As the rebuttal points out, Cariulo et al., 2017 does show that 2B7 and MAB2166 are similar antibodies (Fig. S4 AB ii and iii), while total HTT antibody HDB4ED10 is very different from them (Fig. S5A) - I disagree with the rebuttal statement regarding HDB4ED10 page 10 citing Cariulo et al., 2017. Ellerby's Cong et al., 2011 publication shows both K9 and K444 acetylation confirming Thompson et al. and Jeong et al. 2009 papers. The current authors are not showing a reduction of full-length HTT levels in cells co-expressing TBK1 in figure 2C and 2E detected by Western analysis using MAB2166 - Jeong et al. used MAB5490 to avoid this issue (as the authors did in R3), which might be an option for the authors, or they might choose anti-HTT D7F7 as used for the IP in 2G - detection with MAB2166 shows the reduction dependent on D7F7 IP in this case. In the body of the paper, figures 2C and E should be reprobed with true "total" HTT antibodies which will reflect the author's point of TBK1-mediated reduction in HTT better than MAB2166, which has failed to do so. While I do appreciate the rebuttal figure R5 and understand the authors failed to observe acetylation of K9 by their mass spectrometry analysis, the fact is that it has been observed in the past by others, making 2B7 a poor choice for Singulex analysis of HTT with regards to its

autophagic clearance as it underestimates total HTT levels. Also, R5's inconsistency with Thompson or Ellerby K9 acetylation data does not speak to K444 acetylation or MAB2166 reactivity. As Chaibva et al., 2016 PMID: 27463137 have found that acetylation of HTT N17 alters HTT oligomerization and association with lipids, perhaps the divergence between R5 and Thompson/Ellerby's mass spectrometry K9 acetylation data might reflect differences in sample preparation, the use of HDAC class I/II and class III inhibitors with cell lysis prepared for mass spectrometry (which I do not see in the methods section), the isolation process and the solubility of the species digested, as acetylation may contribute to creation of an oligomerized phosphorylated autophagic clearance intermediate, which may accumulate and aggregate more than unmodified HTT if left uncleared. Figures 1, 2A/B,3,5,6, and 7 all use MAB5492. EV3I-J and EV4C use D7F7, and R3 uses MAB5490 - I applaud these choices.

Responses to referee's comments

Referee #3:

I thank the authors for their careful rebuttal. My concerns have largely been met.

Here are my comments regarding the revised paper and the rebuttal.

General summary and opinion about the principal significance of the study, its questions and findings:

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Response: We agree with the referee that our findings are aligned with previous findings on IKK kinase complex. We would like to point out some key differences between our approach and findings; 1) in this work, TBK1 was identified through an in vitro kinase screening; and 2) we show that TBK1 phosphorylates both mutant and wildtype HTT with high efficacy. While this study was ongoing, Ochaba et al., 2019 showed that knockdown of IKKβ reduces S13 phosphorylation of both wildtype and mutant HTT in animal models of HD. Although they did not assess whether overexpression or activation of IKKβ in mutant HTT expressing models indeed increase HTT S13 phosphorylation, inhibits its aggregation or HTT-induced neurodegeneration, we agree that their findings are consistent with our observations using TBK1.

The manuscript under review beautifully extends this previously published work by showing that similar to IKKbeta, its homologous IKK family member TBK1, a kinase very important in the regulation of autophagy, can also phosphorylate HTT S13 associated with reduced HTT abundance and independent of HTT phosphorylation can also activate its clearance by autophagy leading to reduced mutant HTT aggregate formation. This comprehensive and important manuscript strongly supports the future investigation of TBK1 as a therapeutic target for the treatment of HD, as it demonstrates that TBK1 suppresses mutant HTT-induced toxicity and pathology in HD models. As the HTT protein has been shown to be a scaffold for selective autophagy (Ochaba et al., 2014, Rui et al. 2015), a process in which it may be cleared by the lysosome, the manuscript under review is an exciting update to the autophagy field as well as the HD field, and may reflect a mechanism for upregulation of HTT's autophagic scaffold function in parallel with other TBK1 substrates including HTT-interacting proteins Optineurin and SQSTM1/p62, two autophagic receptor proteins both activated by TBK1-dependent phosphorylation.

Response: We thank the referee for the positive feedback on the quality, comprehensive nature or our and significance of our work.

Minor concerns that should be addressed:
Page 3, line 23 and page 4, line 3 should include Thompson et al., 2009.
Response: We have now cited this work as requested.

Page 8, line 9 - two alphas are redundant - delete the word alpha and keep the Greek letter.
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Response: We thank the referee for pointing this out, we have now corrected the spelling.

Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion):
I have no major concerns and believe the manuscript could be published "as is".
Response: We thank the referee for supporting the publication of the manuscript in the current state.

That being said, I encourage but do not require the authors to revise figure 2C-H, figure EV3G-H, figure EV4H, and figure EV6A using different total HTT antibodies. I believe the data in these figures is reproducible and valid, but may be misleading. While these are commonly used anti-HTT monoclonal antibodies, 2B7 and MAB2166 should not be used to quantitate total HTT levels as their epitopes contain lysines that become modified by acetylation (K9 and K444 respectively) that are found on phosphorylated HTT autophagic clearance intermediates relevant to this manuscript (Thompson et al., 2009, Jeong et al., 2009 PMID: 19345187 and Cong et al., 2011 PMID: 21685499). Acetylation may block the epitopes of these monoclonal antibodies so they should not be used to follow HTT clearance. Thompson et al., 2009 shows acetylation of lysine 9 together with phospho-S13 by mass spectrometry in figure 2C and demonstrates that MAB2166 does not recognize the pS13 species being cleared by autophagy shown in figure S4A (reprobed figure 3D which contains an actin control).

Response: We thank the referee for this suggestion. As indicated in our first response we failed to detect K9 acetylation from 9 Huntingtin samples of exon 1 and purified full length HTT from different sources (Fig R5D). Also, several other studies investigating the HTT PTMs have not reported the detection of K9 acetylation on HTT (Harding, Loppnau et al., 2019, Huang, Lucas et al., 2015, Ratovitski, O'Meally et al., 2017). We believe that K9 acetylated HTT species abundance is possibly very low, and thus will not impact the detection of HTT in these experiments. Moreover, there are no published evidence describing the impact of acetylation on the detection of HTT by 2B7 or MAB2166. While Thompson et al., 2009 (Thompson, Aiken et al., 2009), Jeong et al., 2009 (Jeong, Then et al., 2009), and Cong et al., 2011 (Cong, Held et al., 2011) reported the acetylation, they did not indicate its abundance. This being said, we agree with the referee that a more thorough assessment on how PTMs influence antibody-based detection and quantification is very important.

As the rebuttal points out, Cariulo et al., 2017 does show that 2B7 and MAB2166 are similar antibodies (Fig. S4 AB ii and iii), while total HTT antibody HDB4ED10 is very different from them (Fig. S5A) - I disagree with the rebuttal statement regarding HDB4ED10 page 10 citing Cariulo et al., 2017.

Response: In our assays, either Singulex or WB, we have not observed a significant difference in the detection of HTT levels from these three antibodies 2B7, MAB2166, and HDB4ED10, which target different epitopes of HTT.

Ellerby's Cong et al., 2011 publication shows both K9 and K444 acetylation confirming Thompson et al. and Jeong et al. 2009 papers. The current authors are not showing a reduction of full-length HTT levels in cells co-expressing TBK1 in figure 2C and 2E detected by Western analysis using MAB2166 - Jeong et al. used MAB5490 to avoid this issue (as the authors did in R3), which might be an option for the authors, or they might choose anti-HTT D7F7 as used for the IP in 2G - detection with MAB2166 shows the reduction dependent on D7F7 IP in this case.

Response: We have detected acetylation at K444 during the analysis of the PTMs of full-length HTT in 4 of our samples and N-terminal acetylation in all samples shown in Fig R5D. We appreciate the point regarding the disruption of the MAB2166 epitope by possible K444 acetylation, as also hypothesized in Thompson et al., 2009. We have noted from Thompson et al., 2009, figure 4A, B that the presence of K9 acetylation or S13 phosphorylation did not significantly change the sensitivity of

MAB2166 to detect HTT-FL levels. Moreover, MAB2166 and D7F7 were also shown to detect HTT levels similarly from mouse striatum (Franich, Basso et al., 2018). MAB2166 is a commonly used antibody with approximately 260 citations (www.citeab.com); many used it as a pan HTT antibody (Baldo, Sajjad et al., 2018, Gray, Shirasaki et al., 2008, Hung, Maiuri et al., 2018, Macdonald, Tessari et al., 2014, Ooi, Langley et al., 2019, Warby, Chan et al., 2005). However, we acknowledge the importance of the further investigation to directly assess the relative abundance of lysine acetylation at different residues and how this and other PTMs influence HTT detection by these antibodies, which are the two of the most used antibodies for the development of SMCs and MSA assay for detection of HTT from CSF and biofluids a biomarker in commercial and academic setups (Baldo et al., 2018, Fodale, Boggio et al., 2017, Reindl, Baldo et al., 2019) (https://www.criver.com/sites/default/files/resource-files/CHDI-19-detection-of-Huntingtons-disease-biomarkers.pdf).

In the body of the paper, figures 2C and E should be reprobed with true "total" HTT antibodies which will reflect the author's point of TBK1-mediated reduction in HTT better than MAB2166, which has failed to do so.

Response: We thank the referee for the suggestions regarding the true total HTT antibody. We will consider these suggestions during our future experiments; here, as the referee also noted below, we have relied mainly on antibodies MAB5492, D7F7, and MAB5490 to detect the total HTT.

While I do appreciate the rebuttal figure R5 and understand the authors failed to observe acetylation of K9 by their mass spectrometry analysis, the fact is that it has been observed in the past by others, making 2B7 a poor choice for Singulex analysis of HTT with regards to its autophagic clearance as it underestimates total HTT levels. Also, R5's inconsistency with Thompson or Ellerby K9 acetylation data does not speak to K444 acetylation or MAB2166 reactivity. As Chaibva et al., 2016 PMID: 27463137 have found that acetylation of HTT N17 alters HTT oligomerization and association with lipids, perhaps the divergence between R5 and Thompson/Ellerby's mass spectrometry K9 acetylation data might reflect differences in sample preparation, the use of HDAC class I/II and class III inhibitors with cell lysis prepared for mass spectrometry (which I do not see in the methods section), the isolation process and the solubility of the species digested, as acetylation may contribute to creation of an oligomerized phosphorylated autophagic clearance intermediate, which may accumulate and aggregate more than unmodified HTT if left uncleared.

Response: We have not used the HADC inhibitors in our lysis buffer. However, we were able to detect N-terminal acetylation in all our samples (Fig R5D), indicating that acetylation is still preserved in our samples to a detectable level. We have not probed for any oligomerized phosphorylated autophagic clearance intermediates as these oligomeric species of HTT are hard to detect and measure accuratel, as explain in our first response. Additionally, we showed in our recent paper (Chiki, DeGuire et al., 2017) that untagged HTTex1 with site-specific acetylation at K6 or K9 or K16 did not significantly alter the aggregation of mutant Httex1. This work cannot be compared to previous studies by Chaibva et al (Chaibva, Jawahery et al., 2016), where they based their findings on nonselective chemical acetylation of all lysine residues in a model peptide of HTTex1 lacking the last 39 C-terminal residues and containing two non-native lysine residues.

Figures 1, 2A/B,3,5,6, and 7 all use MAB5492. EV3I-J and EV4C use D7F7, and R3 uses MAB5490 - I applaud these choices.
Response: We thank the referee for highlighting this.

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2nd Revision - Editorial Decision 16th Jun 2020

Dear Hilal,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at everything and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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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 field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased 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< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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 (eg 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 description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- 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 vs. unpaired), simple $\chi 2$ tests, 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 values = x but not P values < x;
  - definition of 'center values' 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). 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

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size were determined based on previous studies using similar methodologies.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Animal size was determined based on the previous similar studies.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusion creteria was used.
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.	Randomization procedures were not applied.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Some of the key experiments of phosphorylation and toxicity has been repeated in other labs
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Students t-test and Anova was applied to assess if the data means significantly differ from each other.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	A paired t-test was applied (tail:2) with a normal distribution. Statistical analysis was also performed by to-way ANOVA in some key experiment.
Is there an estimate of variation within each group of data?	No

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog numbers and references for all used antibodies are described in the appendix table S1
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines (HEK293, HEK293T) were obtained from ATCC and routinely checked for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Tbk1 -/-, Tnf α-/- C57BL / 6JRJ were maintaned in EFPL animal facility according to standard procedure. zQ175 HD (strainC57 BL/6J) mouse model tissue was obtained from Jackson laboratory. Rat and mouse primary culture were from strain OFA (Oncins France Strain A) C57BL/6J respectively were used under Switzerland liscence VD2137 and VD3392.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	No live vertibrates were used.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) 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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	All protocols in this study were approved by Cambridge Brain Bank NRES approval 10/H0308/56 and University of Auckland Human Participants Ethics Committee (2008/279 and 011654),
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All the data in the manuscript or the appendix are deposited to the repository "biostudies" accession number S-BSST413 (www.ebi.ac.uk/biostudies/studies/S-BSST413).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's 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).	All sorce data of this manuscript is presented in a source data files document.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting 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 dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA