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Huntingtin proteolysis releases non-polyA fragments that cause toxicity through dynamin 1 dysregulation

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 28 January 2015

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

As you can see below, the referees appreciate the characterization of C-terminal HTT fragments that promotes toxicity via modulating ER stress. However, they also raise a number of issues that would have to be resolved in order for consideration here. A major issue to resolve is the need to provide further data in support of that C-terminal HTT-mediated toxicity is relevant for disease. Should you be able to address the major concerns raised then I would like to invite you to submit a revised version of the manuscript. I should add that it is EMBO Journal policy to allow only a single major round of revision only and that it is therefore important to address the concerns raised at this stage.

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

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

This paper suggests that a C-terminal fragment of huntingtin is formed after its cleavage is toxic and that this amplifies the toxicity of the N-terminal fragment. The background is that mutant huntingtin is known to be cleaved by different proteases and the formation of N-terminal fragments which contain the expanded polyglutamine (polyQ) tract are believed to be the main driver of toxicity. The current study uses an elegant method employing replacement of some of the huntingtin cleavage sequences with TEV protease recognition sites, in order to characterise the consequences of cleavage.
Overall, the data themselves are generally clear. However, I think there are two critical questions that are raised by the paper.

1. I am convinced that excess C-terminal huntingtin can be toxic. The question is whether this is physiologically relevant to the disease or whether this is simply an overexpression artefact. In contrast to the N-terminal fragments, the C-terminal fragments are the same in wild-type and mutant alleles. The main support provided by the authors for the relevance of this is in 2E. However, here the levels of the C-terminal fragments are not increased in the patient brains - if anything they are reduced. The authors state that the excess of the N-terminal fragments in the patients brains is indicative of more cleavage 9and the inference is that this will cause the production of more C-terminal fragments). This is not necessarily correct. Their may be more production of these fragments but their turnover may be rapid enough to result in very low levels at steady-state. Alternatively, there may be no increased cleavage of the mutant allele-products - the excess of the N-terminal fragments in the patients may be because they have polyQ expansions which make them oligomerise-aggregate and such products may have a slower turnover. Alternatively, this may be due to repeat length dependent-alternative splicing causing the formation from N-terminal fragments preferentially from mutant chromosomes, as has been suggested by Bates and colleagues (Proc Natl Acad Sci U S A. 2013 Feb 5;110(6):2366-70).
This issue of overexpression artefact is my major concern, and as this fragment is the same in mutant and wild-type alleles, the authors do need to provide real support that this has likely relevance.

2. The mechanism for the toxicity of the C-terminal fragment is related to dynamin 1. Some of this looks like it is due to effects on endocytosis. However, the authors argue that a major consequence is via effects on dynamin1 on the ER. The problem here is that dynamin 1 has not been previously found on ER. The authors go some way to trying to show dynamin on the ER. Some of this relies of fractionation (6D). Here the authors need to acknowledge that the fractionation is not so pure and that there is is endosome contamination in their ER fraction. The authors have also not assessed plasma membrane in the ER fraction. More careful assessment of different markers in experiments like this would improve the confidence in the result. Personally, I would suggest that the ER-dynamin mechanism could be couched in a slightly speculative tone pointing out the caveats of this being a sole driver of the pathology, as the endocytosis data provide another mechanism that is plausible (though not mutually exclusive).

Comments of a more technical nature.
1. It is possible that the TEV sites alter htt structure and thus inferences about accessibility of sites may be somewhat risky. Furthermore, as different proteases cleave the different sites in vivo, and the concentrations/activities of these proteases will likely differ, it is similarly risky to make strong inferences about the order of cleavages based on the TEV system which is uniform as least as far as the protease is concerned.
2.Fig 3A - nucleus looks fragmented.
3. Statistics are missing from some key experiments like Fig 3A and 5B and C.
4. E6D needs to be interpreted in the context of the dynamin1 being targetted artificially to the ER

Referee #2:

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

The role of proteolysis of the mutant HTT has generally focused on the effect of N-terminal polyQ expanded HTT on cellular toxicity in vitro and vivo. It is well established that along with the N-terminal polyQ containing fragments a number of C-terminal fragments of HTT are generated in cells, mouse models and human post mortem tissue. The biological activity of these fragments has not been studied. It has been shown in the field of apoptosis that the cleavage of caspase substrates generates fragments that can act to accelerate or inhibit the apoptotic process. To determine the affect of the HTT C-terminal fragments the authors found that expression of these fragments that lack the polyQ stretch induce toxicity via dilation of the endoplasmic reticulum and increased ER stress. They show that this may be mediated by modulating dynamin 1 function. This fits nicely with the idea that fragments generated by proteolysis have function in promoting cellular dysfunction and death. The authors produce a very interesting full-length form of HTT with engineered TEV cleavage sites at amino acid 167, 513 and 586. These are cleavage sites that are produced in cellular and mouse models of HD. They found that cleavage at 167 did not occur efficiently unless first cleavage occurred at 513 or 586. This suggests sequential proteolysis may occur. Toxicity in cells appeared to require both events. They found that the both N-terminal and C-terminal fragments are toxic in cells. Particularly of note is the C-terminal fragment from WT or mutant HTT is toxic again likely reflecting a role of this fragment in inducing cell death.

Specific major concerns essential to be addressed to support the conclusions

One explanation of the C-terminal fragment toxicity is it acts as a dominant negative on WT HTT function. It would be reasonable to test if WT HTT presence is required for the affect. This could be assessed in HTT knockout cells or fly deficient in HTT. This would change the interpretation of the effects and may reflect the N-terminal interaction with the C-terminal HTT.

Minor concerns that should be addressed

"Even some sites are subjected to proteolysis by different proteases (e.g.: site 552 is cleaved by caspases 2 and 3)(Goldberg et al, 1996; Hermel et al, 2004; Wellington et al, 1998)." This is true in vitro but caspase-2 may be selective for this site in vivo.

Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)
In the introduction the history/literature of HTT proteolysis could be covered more extensively and accurately.

Referee #3:

Authors reported the role of non-polyQ C-terminal fragments of huntingtin using time- and site-specific control of full length HTT proteolysis. The C-ter fragments generated, that do not contain the polyQ stretch, induced toxicity via dilation of the endoplasmic reticulum (ER) and increased ER stress. C-ter HTT bound to dynamin 1 and subsequently impaired its activity at ER membranes. Their findings support a role for HTT on dynamin 1 function and ER homeostasis.
Although the reviewer cannot believe the C-terminal fragment of HTT has a specific role on the HD pathogenesis because the reviewer believes the common pathomechanism of polyQ diseases, it is interesting paper, because the new role of C-terminal fragment of HTT was identified.

Major points
1) in Fig. 2E, lane 3 shows HD is not different from CTR. Post mortem effect might have strong effect on the result. Add more cases or reduce their statement relevant to the HD pathogenesis.
2) Fig 4: The quantitative analysis should be performed. How many cells have nuclear inclusions and how many cells have ER vacuoaltions and those cells are overlapped or not should be described. If possible, using immunochemical analysis the result for many cells should be shown.
3) Does siRNA on dynamin 1 reduce ER vacuolation by C-HTT?

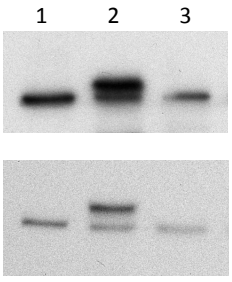
1st Revision - authors' response 30 April 2015

Referee #1:

This paper suggests that a C-terminal fragment of huntingtin is formed after its cleavage is toxic and that this amplifies the toxicity of the N-terminal fragment. The background is that mutant huntingtin is known to be cleaved by different proteases and the formation of N-terminal fragments which contain the expanded polyglutamine (polyQ) tract are believed to be the main driver of toxicity. The current study uses an elegant method employing replacement of some of the huntingtin cleavage sequences with TEV protease recognition sites, in order to characterise the consequences of cleavage.
Overall, the data themselves are generally clear. However, I think there are two critical questions that are raised by the paper.

1. I am convinced that excess C-terminal huntingtin can be toxic. The question is whether this is physiologically relevant to the disease or whether this is simply an overexpression artefact.

We understand reviewer's comment about the physiological relevance and especially about the possibility of overexpression artifact. To circumvent this possibility, we carefully monitored expression of the different constructs for example, in figures 1B and 1C, we focused on the cleavage mechanism. In this case, cells were transfected and we analyzed whole cell extracts by WB to investigate the cleavage process. We privileged conditions allowing high expression to ensure detection of small fragments. Careful quantification gave a ratio of transfected/endogenous HTT of about 12 to18 fold depending of the various constructs.
In toxicity assays such as figure 1D, 2E and 2F, we electroporated cells and followed the fate of mCherry fluorescent cells over 24hrs 1day post-transfection. To analyze the level of expression by WB in such conditions, we selected transfected cells by FACS, based on the expression of the fluorescently tagged HTT, and performed WB analyses. This way we can correlate the level of expression to cell death that is assessed by time-lapse videomicroscopy on cells expressing fluorescently tagged HTT. As seen below the expression of various FL-HTT constructs is about 2 to 6 time more relative to endogenous HTT (see below)



WB analysis of HEK cells transfected with mCherry-HTTQ100 and FACS cell sorted for fluorescence and immunoblotted for HTT using mAb 4C8 antibody (epitope 448-453 of HTT) in two independent experiments.

1- Non transfected cells showing endogenous HTT level
2-cells transfected with siRNA-HTT to silence endogenous HTT and express mCherry-HTTQ100.
3-cells transfected with siRNA-HTT to silence endogenous HTT

While a 2 to 6 fold increase in HTT expression could induce toxicity artifacts, several arguments clearly establish that this is not the case. Indeed:

1) Low HTT levels expressing flies recapitulate events observed in cells
Results obtained in cells have been validated in flies. In contrast to cells, expression of full-length HTT or carboxy-terminus fragment is very low. In flies, the carboxy-terminus fragment of HTT is detectable only after immunoprecipitation (Figure E4C) in contrast to the expression of amino-terminus fragments of HTT (flies developed by the Littleton's group; Figure E4B). However, survival of these flies is reduced compared to flies over-expressing N548-HTTQ128. We show 2 lines (Figure 5D) but obtained similar results for 3 additional fly lines.

2) Importantly, when all the different full-length constructs are expressed in cells and flies, the toxicity due to the C-ter fragment is observed only when protein cleavage is induced. Therefore the differential toxicity observed between non-cleaved HTT and double cleaved HTT is due to the release of the C-ter form N to C intramolecular interaction and cannot be due to an overexpression

artifact as all the constructs are strictly expressed at the same levels.

3) Importantly, the cellular phenotype of vacuolation is observed in mouse brains from full-length knock-In mice as well as in astrocytes derived from HD iPSC from HD patients (Juopperi et al, 2012). This is discussed in the Ms page 20.

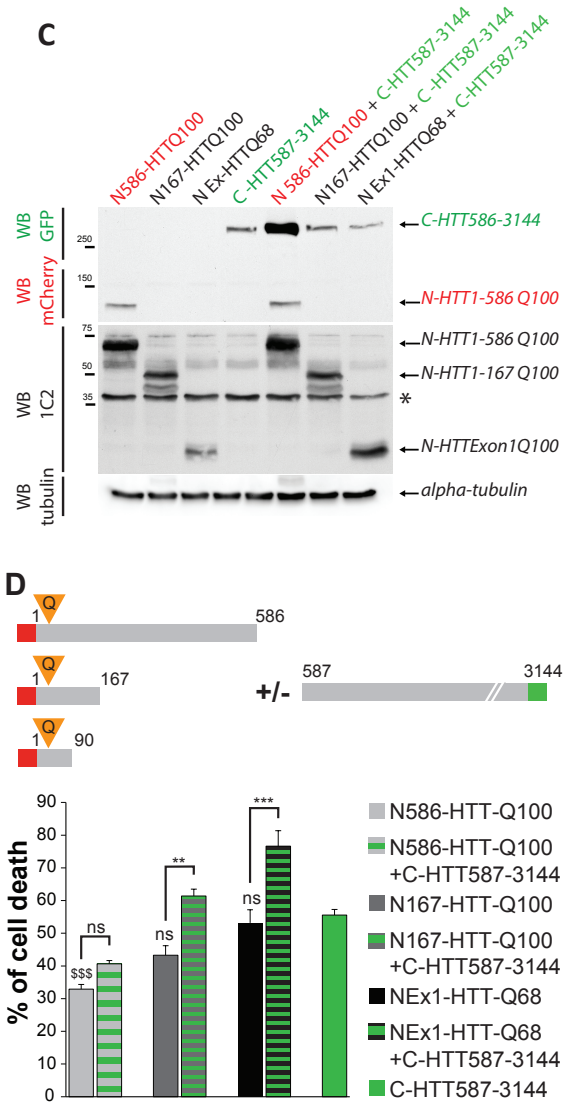
In contrast to the N-terminal fragments, the C-terminal fragments are the same in wild-type and mutant alleles. The main support provided by the authors for the relevance of this is in 2E. However, here the levels of the C-terminal fragments are not increased in the patient brains - if anything they are reduced. The authors state that the excess of the N-terminal fragments in the patients brains is indicative of more cleavage and the inference is that this will cause the production of more C-terminal fragments. This is not necessarily correct. Their may be more production of these fragments but their turnover may be rapid enough to result in very low levels at steady-state. Alternatively, there may be no increased cleavage of the mutant allele-products - the excess of the N-terminal fragments in the patients may be because they have polyQ expansions which make them oligomerise-aggregate and such products may have a slower turnover. Alternatively, this may be due to repeat length dependent-alternative splicing causing the formation from N-terminal fragments preferentially from mutant chromosomes, as has been suggested by Bates and colleagues (ProcNatlAcadSci U S A. 2013 Feb 5;110(6):2366-70). This issue of overexpression artefact is my major concern, and as this fragment is the same in mutant and wild-type alleles, the authors do need to provide real support that this has likely relevance.

In addition to demonstrating the in vivo toxicity of the released C-ter fragment from full length HTT in flies, we tried to address the presence of the different N- and C-ter fragments in patient brains. As raised by reviewer 3, there was a mislabelling of brain sample on the figure (sample 3 was a control). Therefore, we repeated the experiment shown in previous Figure 2E and because of the shortage in one control sample, we focused on 2 control individuals (samples 1-2, age: 53 and 54 years old respectively; post-mortem delay: 22.2 ± 2 h), one HD grade 4 (sample 3), and one grade 3 (sample 4) patients (45 and 82 years old respectively; post-mortem delay: 22.3 ± 0.3 h). We performed Western-blotting analyses using a higher number of anti-HTT antibodies to identify the various fragments. We observed that :

1) The level of full-length polyQ HTT in brain samples from patients is strongly reduced as compared to control individuals. This reduced level of full-length HTT in mutant situation has been reported by many investigators in the field and proposed to be the result of full length HTT proteolysis (see for example (Mende-Mueller et al, 2001).
2) In control situation, we find the presence of full length HTT as well as a large N-ter fragment of 70 kDa and a large C-ter fragment of approximately 250 kDa.
3) In patient samples, although there is a lower level of full length HTT in pathological situation, we observe not only the presence of the large C-ter fragment but also the presence of small N-ter fragments that are not present in the control situation. This is in agreement with our previous results and with previous studies (Gafni & Ellerby, 2002; Goldberg et al, 1996; Hermel et al, 2004; Kim et al, 2006; Kim et al, 2001; Landles et al, 2010; Lunkes et al, 2002; Mende-Mueller et al, 2001; Miller et al, 2010; Wang et al, 2008; Wellington et al, 2002). We agree that we cannot exclude that the observed high level of these small N-ter fragments could be due in part from alternative splicing or a slow turnover of the small N-ter fragments (see result section pages 8 and discussion pages 18 & 19 about the physiological relevance)

Nevertheless, the new important findings (new Figure 2C) reported here in the revised version are the immunoprecipitation experiments performed using post-mortem brain samples. We found that in the control situation, the large N-ter fragments (70kDa) are able to interact with the 250 kDa C-ter fragment (mimicking the data obtained with a single TEV cleavage at position 586). In contrast, in the mutant situation, the smaller 35-55kDa N-ter fragments produced specifically in disease do not interact with the large 250 kDa C-ter fragment. Therefore, we conclude that in brain samples from HD patients, although they are detected at lower levels than in control individuals, the large 250 kDa C-ter fragments are free from any HTT intramolecular interaction. These findings suggest that the release of C-ter fragment modeled by the double TEV cleavage is observed in human HD brain samples. Based on the data obtained in cells and in flies, these fragments free of any intramolecular interaction could be toxic.

In support, we showed that co-expression of both N-ter fragment N586-HTT that has an approximate size of 70 kDa stabilizes the C-HTT587-3144 fragment (approximate size of 250 kDa, see panel C and D below and Figure EV2C) and reduces its toxicity.



Panel C and D : Co-expression of C-HTT587-3144 with N586-HTTQ100 leads to increased stabilization of both amino and carboxy-terminus fragments (panel C) whereas the toxicity of (N586-HTTQ100 + C-HTT587-3144 ; panel D) is reduced compared to the toxicity induced by the carboxy-terminus fragment alone (C-HTT587-3144). * is a non specific band.

Therefore, our results strongly argue that the toxicity of the carboxy-terminus fragment depends highly on its association with amino-terminus fragment rather than on its expression level.

We propose, based on the brain samples analysis and our data in cells and flies, that HTT proteolysis, in addition to generate small N-ter fragments that are toxic in the nucleus, releases non-polyQ C-ter fragments that may also be relevant to HD pathogenesis. Determining the physiological relevance of such fragments in disease may lead to new therapeutic strategies. See discussion page 21.

2. The mechanism for the toxicity of the C-terminal fragment is related to dynamin 1. Some of this looks like it is due to effects on endocytosis. However, the authors argue that a major consequence is via effects on dynamin1 on the ER.
The problem here is that dynamin 1 has not been previously found on ER. The authors go some way to trying to show dynamin on the ER. Some of this relies of fractionation (6D). Here the authors

need to acknowledge that the fractionation is not so pure and that there is is endosome contamination in their ER fraction. The authors have also not assessed plasma membrane in the ER fraction.

We thank the reviewer for this comment. We agree that dynamin has not been reported on ER. That is why we assessed the presence of dynamin using various complementary approaches. Regarding this ER fractionation, the ER-enriched fractions did not show presence of cytosolic protein such as tubulin, neither plasma membrane proteins such as AnnexinV, nor mitochondrial chaperone protein such as mitochondrial hsp70. ER fraction was slightly contaminated with endosomal marker EEA1 and TGN138, however, it exhibited a large enrichment in ER-membrane protein such as calnexin (Figure 6D).
We also detected dynamin on ER using super-resolution microscopy and Electronic microscopy. Together, our data suggest a potential location of dynamin 1 at ER membranes. However, we cannot exclude some contamination and refer to the ER membrane preparation as a ER-membrane enrichment (text page 14).

More careful assessment of different markers in experiments like this would improve the confidence in the result. Personally, I would suggest that the ER-dynamin mechanism could be couched in a slightly speculative tone pointing out the caveats of this being a sole driver of the pathology, as the endocytosis data provide another mechanism that is plausible (though not mutually exclusive).

We agree with reviewer's comment, we therefore specifically acknowledge that part of the toxicity mediated by the C-ter fragment could also be mediated through the inhibition of endocytosis. See result section page 17 and discussion page 19 & 20.

Comments of a more technical nature.
1. It is possible that the TEV sites alter htt structure and thus inferences about accessibility of sites may be somewhat risky.

We thank the reviewer for this important experiment and have therefore assessed HTT function when it contains the TEV sites. We based our functional assay on HTT-dependent Golgi reassembly after microtubule (MT) disruption upon nocodazole treatment. Indeed HTT was shown to maintain the structure of Golgi ribbons and to participate into Golgi reassembly after nocodazole treatment (Caviston et al, 2007) (Pardo et al, 2010). This function depends on HTT interaction with dynein/dynactin complex in the N-terminal region of HTT (regions 171-230 and 536-698, located close to the TEV sites). We therefore silenced endogenous HTT in HeLa cells and tested whether re-expressing a siRNA insensitive HTT constructs containing two TEV sites is able to reassemble Golgi ribbons. Whereas the wild-type HTT containing two TEV sites is able to reform intact Golgi apparatus after nocodazole wash out, the polyQ-containing one is unable to do so. We conclude that HTT with two TEV sites remains fully functional on it's dynein/dynactin function (see text pages 5 & 6 and new figure EV1C).

Furthermore, as different proteases cleave the different sites in vivo, and the concentrations/activities of these proteases will likely differ, it is similarly risky to make strong inferences about the order of cleavages based on the TEV system which is uniform as least as far as the protease is concerned.

We agree with reviewer that we cannot exclude that the concentrations/activities, and possibly of the identity of the proteases themselves could be different in vivo. Here, we can only investigate the relative intrinsic accessibility of the site given that all the sites have been designed identically and that we use the same recombinant protease. Therefore, the advantage of this strategy is to determine the accessibility of the site independently of the identity/concentration/activity of the protease and rather focus on the consequences of HTT proteolysis itself. We have modified the text page 6 and 7 when it comes to claim that based on the TEV system, we unraveled a sequential proteolytic cascade.

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2.Fig 3A - nucleus looks fragmented.

The nucleus looked fragmented as the image was deconvolved. Therefore, only the condensed chromatin was detectable. We have modified the figure (new Figure 3A) and replaced the DAPI image by the DAPI non-deconvolved source image.

3. Statistics are missing from some key experiments like Fig 3A and 5B and C.

We thank the reviewer for this comment. We provide quantification with statistics for figure 3A, 5B and 5C (see complete statistical analyses in expanded view items).

4. E6D needs to be interpreted in the context of the dynamin1 being targetted artificially to the ER.

We agree with reviewer that a better explanation was necessary. We have therefore modified the text accordingly, see page 16 and in the legend of new Figure EV5F and EV5G as well as Figure 7B to make it clear that these constructs are "engineered artificially ER-targeted DNM1" constructs.

Referee #2:

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

The role of proteolysis of the mutant HTT has generally focused on the effect of N-terminal polyQ expanded HTT on cellular toxicity in vitro and vivo. It is well established that along with the N-terminal polyQ containing fragments a number of C-terminal fragments of HTT are generated in cells, mouse models and human post mortem tissue. The biological activity of these fragments has not been studied. It has been shown in the field of apoptosis that the cleavage of caspase substrates generates fragments that can act to accelerate or inhibit the apoptotic process. To determine the affect of the HTT C-terminal fragments the authors found that expression of these fragments that lack the polyQ stretch induce toxicity via dilation of the endoplasmic reticulum and increased ER stress. They show that this may be mediated by modulating dynamin 1 function. This fits nicely with the idea that fragments generated by proteolysis have function in promoting cellular dysfunction and death. The authors produce a very interesting full-length form of HTT with engineered TEV cleavage sites at amino acid 167, 513 and 586. These are cleavage sites that are produced in cellular and mouse models of HD. They found that cleavage at 167 did not occur efficiently unless first cleavage occurred at 513 or 586. This suggests sequential proteolysis may occur. Toxicity in cells appeared to require both events. They found that the both N-terminal and C-terminal fragments are toxic in cells. Particularly of note is the C-terminal fragment from WT or mutant HTT is toxic again likely reflecting a role of this fragment in inducing cell death.

Specific major concerns essential to be addressed to support the conclusions

One explanation of the C-terminal fragment toxicity is it acts as a dominant negative on WT HTT function. It would be reasonable to test if WT HTT presence is required for the affect. This could be assessed in HTT knockout cells or fly deficient in HTT. This would change the interpretation of the effects and may reflect the N-terminal interaction with the C-terminal HTT.

We agree that this is an important experiment. Indeed, since the C-ter fragment when expressed alone induced some toxicity, we tested the possibility that this could occur through a dominant negative effect on full length endogenous HTT that is present in cells. We therefore expressed FL-HTT167/586TEV-Q23 and -Q100 constructs in cells silenced or not for endogenous HTT and induced cleavage by SNIPer TEV activation. We found no difference in the toxicity upon HTT double proteolysis whether endogenous HTT is present or down regulated (New Figure EV2D). Therefore it is unlikely that toxicity induced by the C-ter fragment occurs through a dominant negative effect on wild type endogenous full length HTT. See page 9 and new figure EV2D.

Minor concerns that should be addressed

"Even some sites are subjected to proteolysis by different proteases (e.g.: site 552 is cleaved by caspases 2 and 3)(Goldberg et al, 1996; Hermel et al, 2004; Wellington et al, 1998)." This is true in vitro but caspase-2 may be selective for this site in vivo.

We thank the reviewer for this comment. Indeed, there is strong evidence that caspase-2 (and not 3) is the caspase that cleaves HTT at position 552 in vivo (Hermel et al, 2004). Therefore the argument that a site could be cleaved by two different caspases was not accurate anymore and has therefore been removed from the text. In contrast, we added the example that proteolysis of HTT at 586 is not mediated by Caspase 6 in vivo (Gafni et al, 2012).

Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)
In the introduction the history/literature of HTT proteolysis could be covered more extensively and accurately.

We thank the reviewer for this suggestion. We tried to cover more accurately the literature of HTT proteolysis. We tried to do as best as we can and rely on the editor's discretion to validate these additional references according to the space requirement. We also corrected some references that were missing or were not accurately reported (e.g.: HTT metalloproteinase (Miller et al, 2010), proteolysis inhibition and toxicity (Miller et al, 2010; Wellington et al, 2000), lysosomal proteases (Kim et al, 2006), site 586 (Gafni et al, 2012).

Referee #3:

Authors reported the role of non-polyQ C-terminal fragments of huntingtin using time- and site-specific control of full length HTT proteolysis. The C-ter fragments generated, that do not contain the polyQ stretch, induced toxicity via dilation of the endoplasmic reticulum (ER) and increased ER stress. C-ter HTT bound to dynamin 1 and subsequently impaired its activity at ER membranes. Their findings support a role for HTT on dynamin 1 function and ER homeostasis. Although the reviewer cannot believe the C-terminal fragment of HTT has a specific role on the HD pathogenesis because the reviewer believes the common pathomechanism of polyQ diseases, it is interesting paper, because the new role of C-terminal fragment of HTT was identified.

We agree with reviewer 3 that all the polyQ diseases have in common the presence of the polyQ stretch that is the causing mechanism for all these disorders. However, many studies have demonstrated that each disease is different in term of neurodegeneration and phenotype because of the presence of the polyQ stretch in a different protein context. See for example the many studies about protein phosphorylation, acetylation or methylation outside the polyQ stretch that modulate polyQ toxicity in the different polyQ diseases, as well as the evidence that these mutated proteins induce pathogenesis within the context of their " normal" cellular functions (see Zoghby, Pennuto, Yang, Krainc as well our labs). Therefore, and we fully agree with reviewer that the common mechanism for all these disorders is the polyQ stretch, it is possible that fragments outside this polyQ stretch could modify pathogenesis in a disease specific manner. Therefore and as requested by reviewer 3 we tried to add more physiological relevance to our findings. As we could not obtain more samples in sufficient quantity, we repeated and extended our brain sample analysis by performing immunoprecipitations of the different fragments in brain samples from control and patients. See below.

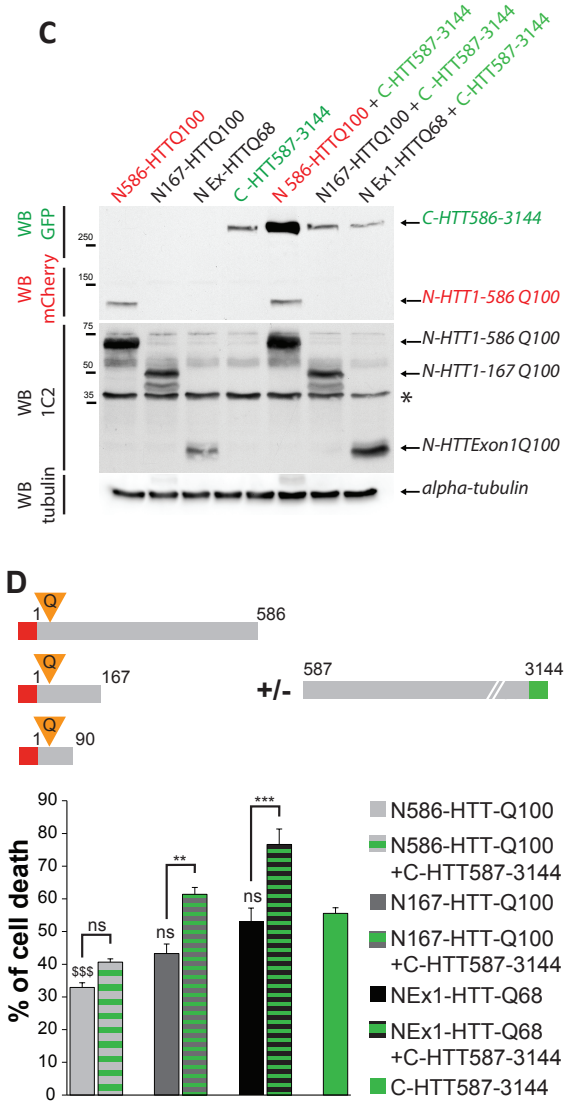
Major points
1) in Fig. 2E, lane 3 shows HD is not different from CTR.
Post mortem effect might have strong effect on the result. Add more cases or reduce their statement relevant to the HD pathogenesis.

We deeply thank reviewer 3 for noticing the mislabeling in previous figure 2E and fully apologize about it. Indeed, as raised by reviewer 3, there was a mislabeling of brain sample on the figure (sample 3 was a control). Therefore, we repeated the experiment shown in previous Figure 2E and because of the shortage in one control sample, we focused on 2 control individuals (samples 1-2, age: 53 and 54 years old respectively; post-mortem delay: 22.2 ± 2 h), one HD grade 4 (sample 3), and one grade 3 (sample 4) patients (45 and 82 years old respectively; post-mortem delay: 22.3 ± 0.3 h). We performed Western-blotting analyses using a higher number of anti-HTT antibodies to identify the various fragments.We observed that :

1) The level of full-length polyQ HTT in brain samples from patients is strongly reduced as compared to control individuals. This reduced level of full-length HTT in mutant situation has been reported by many investigators in the field and proposed to be the result of full length HTT proteolysis (see for example (Mende-Mueller et al, 2001).
2) In control situation, we find the presence of full length HTT as well as a large N-ter fragment of 70 kDa and a large C-ter fragment of approximately 250 kDa.
3) In patient samples, although there is a lower level of full length HTT in pathological situation, we observe not only the presence of the large C-ter fragment but also the presence of small N-ter fragments that are not present in the control situation. This is in agreement with our previous results and with previous studies (Gafni & Ellerby, 2002; Goldberg et al, 1996; Hermel et al, 2004; Kim et al, 2006; Kim et al, 2001; Landles et al, 2010; Lunkes et al, 2002; Mende-Mueller et al, 2001; Miller et al, 2010; Wang et al, 2008; Wellington et al, 2002). We agree that we cannot exclude that the observed high level of these small N-ter fragments could be due in part from alternative splicing or a slow turnover of the small N-ter fragments (see result section pages 8 and discussion pages 18 & 19 about the physiological relevance)

Nevertheless, the new important findings (new Figure 2C) reported here in the revised version are the immunoprecipitation experiments performed using post-mortem brain samples. We found that in the control situation, the large N-ter fragments (70kDa) are able to interact with the 250 kDa C-ter fragment (mimicking the data obtained with a single TEV cleavage at position 586). In contrast, in the mutant situation, the smaller 35-55kDa N-ter fragments produced specifically in disease do not interact with the large 250 kDa C-ter fragment. Therefore, we conclude that in brain samples from HD patients, although they are detected at lower levels than in control individuals, the large 250 kDa C-ter fragments are free from any HTT intramolecular interaction. These findings suggest that the release of C-ter fragment modeled by the double TEV cleavage is observed in human HD brain samples. Based on the data obtained in cells and in flies, these fragments free of any intramolecular interaction could be toxic.

In support, we showed that co-expression of both N-ter fragment N586-HTT that has an approximate size of 70 kDa stabilizes the C-HTT587-3144 fragment (approximate size of 250 kDa, see panel C and D below and Figure EV2C) and reduces its toxicity.



Panel C and D : Co-expression of C-HTT587-3144 with N586-HTTQ100 leads to increased stabilization of both amino and carboxy-terminus fragments (panel C) whereas the toxicity of (N586-HTTQ100 + C-HTT587-3144 ; panel D) is reduced compared to the toxicity induced by the carboxy-terminus fragment alone (C-HTT587-3144). * is a non specific band.

Therefore, our results strongly argue that the toxicity of the carboxy-terminus fragment depends highly on its association with amino-terminus fragment rather than on its expression level.

We propose, based on the brain samples analysis and our data in cells and flies, that HTT proteolysis, in addition to generate small N-ter fragments that are toxic in the nucleus, releases non-polyQ C-ter fragments that may also be relevant to HD pathogenesis. Determining the physiological relevance of such fragments in disease may lead to new therapeutic strategies. See discussion page 21.

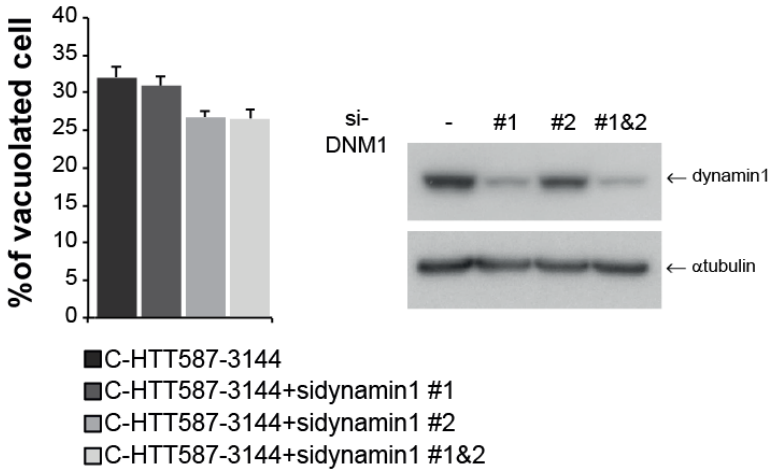
2) Fig 4: The quantitative analysis should be performed. How many cells have nuclear inclusions and how many cells have ER vacuoaltions and those cells are overlapped or not should be described. If possible, using immunochemical analysis the result for many cells should be shown.

We agree that a quantitative analysis is missing. We therefore analyzed the EM images more carefully. We found that in the knock-in mouse model of HD (HdhQ111/Q111), 4.3% of striatal neurons exhibit nuclear aggregates (10/233), while no aggregates could be observed in WT Q7/Q7 mice (total number of neurons scored n= 225 in $Hdh^{Q7/Q7}$ control mice, n=233 in $Hdh^{Q111/Q111}$ mutant mice).

We next performed analysis of the ER ultrastructure of striatal cells in WT and mutant aged matched mouse. We provide below the detailed quantification performed on 3 mice for each genotype. This quantification shows that there is a significant increase in the number of neurons containing vacuoles (39,1%) as compared to control mice (20,4%; unpaired t test p=0.0068). These data are provided on the new graph in figure 4B that illustrates the significant increase in ER-derived vacuoles in HD knock-in mice.

		non swollen	swollen	total striatal neuron	%	mean	SEM
hdhQ7/Q7	mouse nb 1072	64	15	79	0,189873		
	mouse nb 1073	54	10	64	0,15625		
	mouse nb 1074	60	22	82	0,268293	20%	0,033
hdhQ111/Q111	mouse nb 1075	47	27	74	0,364865		
	mouse nb 1076	54	35	89	0,393258		
	mouse nb 1077	41	29	70	0,414286	39%	0,014

3) Does siRNA on dynamin 1 reduce ER vacuolation by C-HTT?



As suggested by the reviewer 3, we used siRNA on dynamin1 and assessed the level of vacuolated cells expressing the C-HTT587-3144-GFP. We used two distinct siRNA targeting dynamin1, alone or together and performed the presented experiments twice in triplicate (total number of cells counted per condition is between 347 and 421 cells). We could not detect significant differences in C-ter HTT-induced vacuolation between cells that contain endogenous level of dynamin1 and cell in which dynamin 1 is down regulated. One possibility could be that the remaining dynamin 1 is sufficient to mediate vacuolation, as it is difficult to remove all dynamin 1 from cells. Alternatively, other dynamin isoforms may also participate in the process as dynamin up regulation has been reported for some dynamin knock out mice. We agree it would be very interesting to test whether C-ter HTT is still toxic in cells that are triple knock out for dynamin1, 2 and 3 (Park et al, 2013) but we believe this is out of the scope from the current study.

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Additional Correspondence 28 May 2015

Thank you for submitting your manuscript to The EMBO Journal. Your revised version has now been re-reviewed by the referees and their comments are provided below. As you can see, the referees appreciate the introduced changes and support publication here.

There are just a few minor comments that needs to be addressed:

Referee #1:

- Suggestion to add a paragraph in the discussion to mention the caveats of the overexpression approach. I agree with this

- Suggestion to remove Figure 2C. I think it would be nice to keep this panel in as I do think it adds insight. As long as you are careful in the interpretations and discussion then that should be fine

Referee #3:

- Suggestion to change the title and remove reference to Dynamin1. I would prefer to leave the Dynamin1 in the title as it is an important part of the paper and I find there is enough data provided to support this mechanism.

I am happy to discuss the issues further if needed. You can send me the modified word file by email and we will replace it.

REFEREE REPORTS

Referee #1

This is a revision of a paper reporting toxicity of a C-terminal fragment of huntingtin. I believe that the fragment is toxic above a certain level of expression. As mentioned in the earlier review, the key question is whether the levels observed in any of the systems employed are physiologically relevant - do humans have similar levels of these fragments? In the cell models there is clearly overexpression and while the levels of the fragments appear low in the fly experiments there may be other reasons for this - e.g. cell death of the cells with the fragments and consequent removal. The new data in 2c are not convincing - while the HD patients appear to have a lack of interaction of the of the C terminal fragment with the larger N-terminal fragments, I cannot see that the experiment presented is conclusive, since the total amount of full-length htt as well as the amounts of the larger N-terminal fragments are much less in the patients - thus the apparent failure to see an interaction may simply be a sensitivity issue.
Otherwise, the revisions have been carefully done.

My overall impression is that the paper presents a daring hypothesis for a process involved in Huntington's disease pathogenesis. This is based on elegant approaches and some very nice experiments. The weakness of the paper is the question of physiological relevance. However, this is a caveat in many studies and I feel that this hypothesis should be aired as it is interesting and potentially important. My suggestion is that the authors introduce a paragraph in the discussion and mention the caveats of their approaches, including the overexpression issues, so that this model is presented with some caution. Caveats should also include discussion of the aberrant splicing mentioned earlier from Bates' group, and its potential contribution to pathogenesis. I also suggest removing Figure 2C, which does not really add much, given its caveats and the fact that only 2 cases and 2 controls have been studied - the cases are also late stage HD patients where most of the relevant parts of the brain are missing in the cases, further complicating the interpretation.

Referee #2

As stated in the first review

The role of proteolysis of the mutant HTT has generally focused on the effect of N-terminal polyQ expanded HTT on cellular toxicity in vitro and vivo. It is well established that along with the N-terminal polyQ containing fragments a number of C-terminal fragments of HTT are generated in cells, mouse models and human post mortem tissue. The biological activity of these fragments has not been studied. It has been shown in the field of apoptosis that the cleavage of caspase substrates generates fragments that can act to accelerate or inhibit the apoptotic process. To determine the affect of the HTT C-terminal fragments the authors found that expression of these fragments that lack the polyQ stretch induce toxicity via dilation of the endoplasmic reticulum and increased ER stress. They show that this may be mediated by modulating dynamin 1 function. This fits nicely with the idea that fragments generated by proteolysis have function in promoting cellular dysfunction and death. The authors produce a very interesting full-length form of HTT with engineered TEV cleavage sites at amino acid 167, 513 and 586. These are cleavage sites that are produced in cellular and mouse models of HD. They found that cleavage at 167 did not occur efficiently unless first cleavage occurred at 513 or 586. This suggests sequential proteolysis may occur. Toxicity in cells appeared to require both events. They found that the both N-terminal and C-terminal fragments are toxic in cells. Particularly of note is the C-terminal fragment from WT or mutant HTT is toxic again likely reflecting a role of this fragment in inducing cell death.

The authors have addressed the issues of my previous review and points raised by the other reviewers.

Referee #3

The authors responded to the reviewer's comments well.
However, the mechanism related to dynamin1 is not so strongly supported and the title shoudl be modified to "Huntingtin proteolysis causes toxicity through non-polyQ C-terminal fragment" or others.

Authors' response 11 June 2015

Referee #1:

- Suggestion to add a paragraph in the discussion to mention the caveats of the overexpression approach. I agree with this

See page 19 in the discussion.

The reviewer 1 asked to mention again the aberrant splicing as a possible cause for toxicity in HD. The fact that aberrant splicing can be important in HD was already mentioned page 8. I am not sure that emphasizing more this hypothesis (that has not been reproduced by others so far) make sense in a paper on proteolysis. I hope you agree with this.

- Suggestion to remove Figure 2C. I think it would be nice to keep this panel in as I do think it adds insight. As long as you are careful in the interpretations and discussion then that should be fine

See page 9 and page 19 to make sure it is not overstated.

Referee #3:

- Suggestion to change the title and remove reference to Dynamin1. I would prefer to leave the Dynamin1 in the title as it is an important part of the paper and I find there is enough data provided to support this mechanism.

Thank you again for your support. Interestingly, the reviewer suggest a title that i wrote previously but finally changed for a more cautious one regarding the non-polyQ fragments: I don't know whether such title would not be better : here is a suggestion:

Huntingtin proteolysis releases non-polyQ fragments that cause toxicity through dynamin 1 dysregulation

