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Loss of FBXO7 (PARK15) results in reduced proteasome activity and models a parkinsonism-like phenotype in mice

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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 14 January 2016

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

As you can see from the reports, both referees appreciate the insights gained from the analysis on FBXO7. However, they also find that certain aspects of the study need to be improved in order to consider publication here. The referees raise similar concerns and find that further analysis is needed to strengthen the findings on the role of FBXO7 on PSMA2 ubiquitination and proteasomal assembly. Should you be able to extend the analysis along the lines indicated by the referees then I would like to invite you to submit a revised version of the manuscript, addressing the comments of the reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and that it is therefore important to address the raised concerns at this stage.

REFEREE REPORTS

Referee #1:

The authors applied sophisticated genetic approaches in mice to explore the function of Fbxo7, a gene whose mutations are associated with familial Parkinsonism. They studied the mouse phenotypes after whole-body deletion of Fbxo7, or tissue specific deletion of Fbxo7 in either forebrain neurons or dopaminergic neurons. Their observation of early motor defects in these mice is of appreciable interest and suggests that these mouse models could serve as tools to further study

the function of Fbxo7 and how its deficiency may lead to Parkinsonism in humans. On the other hand, their analysis of Fbxo7's targets and effects on proteasome assembly are premature and not convincing. These questions clearly requite further work.

Specific Concerns:
1. In figure 1C, why are Fbxo7 proteins almost not detectable in the heterozygous mice (Fbxo7+/-)?
2. The motor defects in forebrain neuron specific deletion of Fbxo7 are quite intriguing. However, it would be better to also perform assays for brain neurons. The question here is whether the motor defects are early and selective or are accompanying or subsequent to other neurological defects (e.g., memory, sensory...).
3. The authors reported an interesting and clear, but surprising interaction between Psma2 and the Ubl domain of Fbxo7, as well as some evidence of ubiquitination of Psma2 by K63-linkage by Fbxo7. However, the claimed function of Fbxo7 in 26S assembly (i.e., decreases in 26S and increases in 20S contents after Fbxo7 deletion (Fig 5d-e)) are not at all convincing. Also, three is no connection between this putative role of Fbxo7 and the development of dopamine deficiency and Parkinsonism. Therefore, unlike the nice genetic and phenotypic analysis presented, these biochemical studies of Fbxo7 and proteasomal regulation appear premature and await further clarification and more thorough investigation.
4. It is very surprising that the Ubl-domain interacts with PSMA2, since ubiquitin-like domains are known to interact with 19S subunits (Rpn13 and Rpn1). This point should be discussed critically. Do other Ubl domains bind to PSMA2 or could there be other functional domains in the Ubl region?
5. Some experiments could be explained more fully and precisely. For example, p8, by ubiquitination assay, the authors seem to mean overexpression and not a biochemical assay with pure components. This is a major difference!
6. Are there any precedents for F-box proteins generating K63 chains - If so, it is interesting which E2 is utilized, since CDC34 (the standard E2) generates K48 chains.
7. Also, is there any evidence for PSMA2 ubiquitination normally, and not just upon Fbxo7 overexpression, or whether intact proteasomes are ubiquitinated and not just the free PSMA2 subunit.
8. Why is it significant that siRNA for PSMA2 "phenocopied" siRNA for Fxo7 since there is no evidence for degradation of this subunit? The data on Fig 5 d-,h are not very clear or convincing as to whether the activity or amounts of the particles are reduced. How were data in 5a- controlled?

Minor points:
1. Often, the language can be more specific. For example, P15, line 11-12: says nothing. Line 20: "Proteasomal subunits are postsynthetically modified".

Referee #2:

This manuscript by Vingill et al. describes the consequences of the systemic and conditional loss of FBXO7 on motor behaviour and neuropathology in mice. At the molecular level, the authors identify PSMA2, a proteasomal subunit, as a new FBXO7 interactor. They then suggest that FBXO7 affects proteasome assembly, possibly through PSMA2 ubiquitination.

General comments:

The paper is very well written, and most of the experiments appear to be well controlled. The phenotype of the FBXO7 mice is interesting, and in itself, justifies a publication in EMBOJ. However, the solidity of the molecular biology data (regarding the effect of FBXO7 deficiency on PSMA2 ubiquitination and proteasomal assembly in particular) is less convincing and some experiments should be repeated prior to publication.

Specific comments:

1. Do the authors know whether or not L-DOPA rescues the abnormal phenotype in the FBXO7 knockout mouse?
2. In Figure 2, the apoptosis data should be confirmed by other methods than TUNEL (e.g. caspase cleavage, PI staining).

3. Most of the biochemistry experiments have been performed using over-expressed proteins. Whenever possible, the authors should repeat the experiments with endogenous proteins (e.g. co-IP and fractionation in Figure 3).
4. Is the FBXO7/PSMA2 interaction a direct interaction? The authors should perform in vitro pull down assays (with in vitro translated or recombinant proteins).
5. In Figure 3e, is that FBXO7 isoform 2 which is pull downed with the Ubl domain of RAD23B? If so, how do the authors reconcile that with the fact that isoform 2 lacks the Ubl domain and thus its ability to bind the proteasome?
6. All together, the ubiquitination data are not completely convincing (e.g. small magnitude of the effect in Figures 4b, S4c, and Figures 5d, f, g, h in particular). These experiments should be repeated and quantified.
7. Ideally the biochemistry experiments should be repeated in patient's cells carrying an FBXO7 mutation.

Minor comments:
1. Page 7, the reference 18 is not recent (2009)
2. The authors should make sure they don't make any overstatements (e.g. page 9, KD of FBXO7 in HEK293T cells led to a "marked" increase....).
3. Loading controls are missing in some biochemistry figures (e.g. Figure 5)

1st Revision - authors' response 13 June 2016

We were pleased with the positive response and critique of the reviewers to our study. They helped us greatly to improve the manuscript. In the revised manuscript, we have addressed all of the reviewers' concerns, mostly with new experiments. Here, I will outline our response to the reviewers' comments.

Referee #1:

The authors applied sophisticated genetic approaches in mice to explore the function of Fbxo7, a gene whose mutations are associated with familial Parkinsonism. They studied the mouse phenotypes after whole-body deletion of Fbxo7, or tissue specific deletion of Fbxo7 in either forebrain neurons or dopaminergic neurons. Their observation of early motor defects in these mice is of appreciable interest and suggests that these mouse models could serve as tools to further study the function of Fbxo7 and how its deficiency may lead to Parkinsonism in humans. On the other hand, their analysis of Fbxo7's targets and effects on proteasome assembly are premature and not convincing. These questions clearly requite further work.

Specific Concerns:
1. In figure 1C, why are Fbxo7 proteins almost not detectable in the heterozygous mice (Fbxo7+/-)?
- *We have repeated the immunoblotting experiment and replaced **Fig1C** with a new image of good quality.*

2. The motor defects in forebrain neuron specific deletion of Fbxo7 are quite intriguing. However, it would be better to also perform assays for brain neurons. The question here is whether the motor defects are early and selective or are accompanying or subsequent to other neurological defects (e.g., memory, sensory...).
- *The FBXO7fl/fl;Nex-Cre mouse is indeed quite interesting since the mice not only display motor impairment but also show signs of spasticity and stereotypic behavior. The latter is characterized by circling behavior, which made it impossible to do memory experiments (object recognition test e.g. completely failed as the mice start circling between objects). The mouse is also extremely jumpy and agile, which makes e.g. the hot plate test impossible to interpret.*

3. The authors reported an interesting and clear, but surprising interaction between Psma2 and the Ubl domain of Fbxo7, as well as some evidence of ubiquitination of Psma2 by K63-linkage by Fbxo7. However, the claimed function of Fbxo7 in 26S assembly (i.e., decreases in 26S and increases in 20S contents after Fbxo7 deletion (Fig 5d-e)) are not at all convincing.

- *To clarify and support our current data, we have repeated and quantified the experiments to demonstrate the reproducibility of the results and included those in **Fig 5E** and in **Fig 5H**. In addition, we have performed activity assays of fractionated lysates from control and FBXO7 RNAi cells. This method was also successfully used to demonstrate loss of proteasome function by Bedford and colleagues (Bedford, Hay et al., 2008). Our data show a control activity curve with one major peak reflecting the 26/30S proteasome. Upon FBXO7 RNAi, we observe the appearance of a second peak, reflecting free 20S proteasomes, supporting the notion that FBXO7 is required for proteasome assembly or stability. These results are now included in **Fig 5G**.*

Also, three is no connection between this putative role of Fbxo7 and the development of dopamine deficiency and Parkinsonism. Therefore, unlike the nice genetic and phenotypic analysis presented, these biochemical studies of Fbxo7 and proteasomal regulation appear premature and await further clarification and more thorough investigation.

- *This is definitely a point that we will address in the future. To make a connection between FBXO7's role in proteasome function, the decrease in dopamine concentration and parkinsonism is a complex goal. The literature on the connection between dopaminergic neurons and proteasomal function is sparse and requires further basic research. The pursuit will entail an entire series of experiments in addition to the generation of new mouse models, which would be beyond the scope of the current study.*

4. It is very surprising that the Ubl-domain interacts with PSMA2, since ubiquitin-like domains are known to interact with 19S subunits (Rpn13 and Rpn1). This point should be discussed critically. Do other Ubl domains bind to PSMA2 or could there be other functional domains in the Ubl region?

- *You raised an interesting point that generated some doubts about domain assignment of FBXO7 in the literature. While a few papers assigned a Ubl domain to the N-terminus of FBXO7, our investigation led us to disagree with this domain name. We have examined FBXO7 using domain search programs, which revealed a ubiquitin-related domain (aa 2-95) in ENSEMBL. Neither SMART nor SwissProt found a ubiquitin-like domain in FBXO7, but in parkin (positive control). Hence, there appears to be a difference in sequence similarity between Ubl and ubiquitin-related domain, which could explain the difference in binding behavior. We have thus decided to refer to this domain as ubiquitin related domain (UbRD). The Ubl of parkin also appears to be less specialized than other Ubl domains since it mediates both the interaction with the 19S subunit Rpn10 and with ataxin-3. In contrast to the previously characterized Ubl domains the UbRD domain of FBXO7 mediates the interaction with the proteasomal core subunit PSMA2. We have included these observations and studies in the discussion.*
- *We have no knowledge of other Ubl (or UbRD) domains that associate with PSMA2 nor do we know if there are further functional domains within the Ubl (UbRD) domain.*

5. Some experiments could be explained more fully and precisely. For example, p8, by ubiquitination assay, the authors seem to mean overexpression and not a biochemical assay with pure components. This is a major difference!

- *As requested, we have elaborated on the cell-based ubiquitination and correctly referred to the type of assay used in the results.*

6. Are there any precedents for F-box proteins generating K63 chains - If so, it is interesting which E2 is utilized, since CDC34 (the standard E2) generates K48 chains.

- Yes, there are. The F-box proteins beta-TRCP and SKP2 have been shown to attach K63 polyubiquitin chains. We have included these studies in the discussion.
- *In addition, we have included an in vitro ubiquitination assay to show the usage of the E2 enzymes and the successful ubiquitination of PSMA2 by FBXO7. We found that FBXO7-SCF does not use UbcH13 (negative control) but it uses UbcH5c and to a lesser extent UbcH3 (Cdc34) in vitro. Of UbcH5c, it is known that it mediates the transfer of K11, K48 and K63 chains in vitro. This results was included in **Fig 4H**.*

7. Also, is there any evidence for PSMA2 ubiquitination normally, and not just upon Fbxo7 overexpression, or whether intact proteasomes are ubiquitinated and not just the free PSMA2 subunit.

- *There are several mass spectrometry studies of proteasome subunits that have identified lysines on PSMA2 that are ubiquitinated. These studies include analyses of the ubiquitinated proteome and ubiquitinated proteasomes. We have included this information in the discussion. The studies that present data derived from enriched ubiquitinated proteome, likely includes both free and bound PSMA2.*

8. Why is it significant that siRNA for PSMA2 "phenocopied" siRNA for Fxo7 since there is no evidence for degradation of this subunit?

- *We rephrased this paragraph to make the RNAi results clearer. Given that FBXO7 RNAi and PSMA2 RNAi reduce proteasome activity, the results could mean that the absence of the suggested FBXO7-mediated ubiquitination of PSMA2 has the same magnitude of effect on proteasome activity as reduction in PSMA2.*

The data on Fig 5 d-h are not very clear or convincing as to whether the activity or amounts of the particles are reduced.

- *To clarify the data, we have quantified all the results and included further data in **Fig 5** to support our conclusions as elaborated above. We have shown that activity of the purified proteasome holoenzymes is unaltered between WT and FBXO7 KO brains, which indicates that once the proteasomes are fully assembled, they are fully active. But when we examine the entire proteasome pool in the cell, we do see a change in particle distribution both upon FBXO7 RNAi and FBXO7 KO (HEK293T cells and brain). As a result, we find more free core particles and less proteasome holoenzymes. We have reworded this paragraph to clarify the conclusions of these important results.*

How were data in 5a- controlled?

- *We controlled the proteasome activity assay with immunoblotting of the input lysates from empty control vector-transfected cells, or cells, transfected with non-functional RNAis, which are shown in **Fig EV3A, B**. Prior to proteasome activity testing, we have used specific proteasome inhibitors to establish the proper readout of the activity measurements. The latter is standard but we have now included a sentence mentioning the preparative work.*

Minor points:
1. Often, the language can be more specific. For example, P15, line 11-12: says nothing. Line 20: "Proteasomal subunits are postsynthetically modified".

- *As requested, we have specified and elaborated this sentence. We have also elaborated on many other occasions to clarify experiments and statements.*

Referee #2:

This manuscript by Vingill et al. describes the consequences of the systemic and conditional loss of FBXO7 on motor behaviour and neuropathology in mice. At the molecular level, the authors identify PSMA2, a proteasomal subunit, as a new FBXO7 interactor. They then suggest that FBXO7 affects proteasome assembly, possibly through PSMA2 ubiquitination.

General comments:

The paper is very well written, and most of the experiments appear to be well controlled. The phenotype of the FBXO7 mice is interesting, and in itself, justifies a publication in EMBOJ.

- *We thank the reviewer for appreciating our data.*

However, the solidity of the molecular biology data (regarding the effect of FBXO7 deficiency on PSMA2 ubiquitination and proteasomal assembly in particular) is less convincing and some experiments should be repeated prior to publication.

Specific comments:

1. Do the authors know whether or not L-DOPA rescues the abnormal phenotype in the FBXO7 knockout mouse?

- *This is a great question and we have addressed this question using a small 8 month-old cohort of FBXO7; TH-Cre mice. We tried to alleviate the motor symptoms with a levodopa/benserazide mixture. The mice however responded with hyperactivity. This was reminiscent of studies in which MPTP mice treated with levodopa reacted in a similar manner (Nicholas, 2007). This is typical for early dopaminergic denervation hypersensitivity by an assumed upregulation of dopaminergic receptors (or their sensitivity).*

2. In Figure 2, the apoptosis data should be confirmed by other methods than TUNEL (e.g. caspase cleavage, PI staining).

- *As suggested, we have carried out immunohistochemistry of the cortical tissue with the cleaved caspase-3 antibody. Here, we found an upward trend in CC3 signal in the KO cortices, which was not statistically significant. This result is now **Appendix Fig S1C**. We have also looked into the P9 cortices but found no difference between WT and FBXO7 KO cortices. Hence, we conclude that FBXO7 is required for proper neuron function but in the tissue context not absolutely required for survival. The isolated neuron culture system however does suggest a contribution to neuronal integrity and health.*

3. Most of the biochemistry experiments have been performed using over-expressed proteins. Whenever possible, the authors should repeat the experiments with endogenous proteins (e.g. co-IP and fractionation in Figure 3).

- *As suggested, we have repeated the fractionation experiments to examine endogenous FBXO7 both in HEK293T cells and in cortical tissue, which are now included in **Fig 3F** and **Fig EV2D**. We have also tried to include a co-IP. The problem here was the PSMA2 antibodies. The monoclonal antibodies that worked in immunoprecipitation, recognizes human PSMA2 only and did not work on mouse brain lysates. Two polyclonal antibodies that recognized murine PSMA2 generated so much background that we could not confirm the specific immunoprecipitation of PSMA2.*

4. Is the FBXO7/PSMA2 interaction a direct interaction? The authors should perform in vitro pull down assays (with in vitro translated or recombinant proteins).

- *Yes, since we identified this interaction partner in a yeast-two-hybrid screen, the interaction of FBXO7 and PSMA2 is a direct one. However, we also included a new experiment using recombinant proteins to underscore the direct interaction between the two. This result is shown in **Fig 3B**.*

5. In Figure 3e, is that FBXO7 isoform 2 which is pull downed with the Ubl domain of RAD23B? If so, how do the authors reconcile that with the fact that isoform 2 lacks the Ubl domain and thus its ability to bind the proteasome?

- *No, it is not FBXO7 isoform 2, but a non-specific band. We have included better labeling of the **Fig 3G** to present clear results. In our study however, we have shown that specific knockdown of isoform 1 contributes to proteasomal integrity and we suggest this to be the relevant isoform in this context.*

6. All together, the ubiquitination data are not completely convincing (e.g. small magnitude of the effect in Figures 4b, S4c, and Figures 5d, f, g, h in particular). These experiments should be repeated and quantified.

- *As per request we have repeated and quantified the experiments in **Fig 4B, C, D E**, and **Fig 5E, H** included these graphs underscore the reproducibility and reliability of the results. We have also included new fractionation analyses combined with activity measurements in **Fig 5G**. Please refer to reviewer 1, comment #3 and comment #8, bullet point #2.*

7. Ideally the biochemistry experiments should be repeated in patient's cells carrying an FBXO7 mutation.

- *This is indeed a great thought and would be an important set of experiments, although we haven't been able to establish collaborations with labs working on patient material to get access on patients' material.*

Minor comments:

1. Page 7, the reference 18 is not recent (2009)
   - *We have added a more recent reference by Fabre et al., that shows FBXO7 as a proteasome interactor. We however kept the older reference owing to the fact that this study also described the presence of other SCF core complex components at the proteasome.*

2. The authors should make sure they don't make any overstatements (e.g. page 9, KD of FBXO7 in HEK293T cells led to a "marked" increase....).
   - *We removed the "marked" remark.*

3. Loading controls are missing in some biochemistry figures (e.g. Figure 5)
   - *We have included the loading controls in **Fig EV3A, B, C, F, G, J**. We pointed it out in the text. We have also added a missing loading control for **Fig 5F**, which can be found in **Fig EV3H**.*

References:
Bedford L, Hay D, Devoy A, Paine S, Powe DG, Seth R, Gray T, Topham I, Fone K, Rezvani N, Mee M, Soane T, Layfield R, Sheppard PW, Ebendal T, Usoskin D, Lowe J, Mayer RJ (2008) Depletion of 26S proteasomes in mouse brain neurons causes neurodegeneration and Lewy-like inclusions resembling human pale bodies. J Neurosci 28: 8189-98
Nicholas AP (2007) Levodopa-induced hyperactivity in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Mov Disord 22: 99-104

2nd Editorial Decision 07 July 2016

Thank you for submitting your revision to The EMBO Journal. Your revision has now been re-reviewed by referee #2. As you can see below this referee appreciates the introduced changes. Referee #2 still finds the analysis of Fbx7 a bit premature, but is overall supportive.

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

REFEREE REPORT

Referee #2:

The authors have answered most of my comments. Their analysis of Fbxo7 on proteasome assembly is still a little premature in my opinion (the authors may want to state that in the discussion). However the the phenotype of the FBXO7 mice is interesting in itself and I feel that the manuscript is suitable for publication in EMBO J.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Judith Stegmueller
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2015-93585

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the 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 justified
- 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 χ2 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.

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

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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?	For histological analyses an n of three per genotype was pre-chosen to detect a difference of p<0.05. For behavioral testing an n of 10-15 animals was pre-chosen to detect a difference of p<0.05. For biochemical experiments three independent experiments was pre-chosen to detect a difference of p<0.05
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For behavioral studies a test cohort of 8 mice was examined to ensure normality distribution of data and functional testing procedures.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	TH-Cre;fl/fl animals above 40 grams and TH-Cre+ animals below 30 grams at 6 months were excluded from the analysis due to extreme difference in weight. This difference was not anticipated and the decision was made post hoc.
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.	Behavioral testing was done in sequence according to moiuse number, which was assigned randomly. All neuropathological and biochemical analyses were conducted in a blinded manner.
For animal studies, include a statement about randomization even if no randomization was used.	see above
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.	All analyses were done in a blinded manner where the investigator was made unaware of the genotype of the mouse before analyzing the data.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For the study of the conventional FBXO7 knockout mice, the researcher could not be kept blinded due to extreme weight differences. For all other behavioral testing and tissue analyses the investigator was blind to the mice's genotype, even though the phenotype was noticeable during testing procedures.
5. For every figure, are statistical tests justified as appropriate?	Data that were normally distributed were analyzed using one-way ANOVA with a Tukey multiple comparison test post-hoc. For two group,s a two-tailed t-test was used, either paired or unpaired as stated.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	D'Agostino & Pearson omnibus normality test was used to assess normal distribution. If data was not normally distributed, the non-parametric Kruskal Wallis test with post-hoc Dunn's multiple comparison was used to assess statistical significance between groups. For two groups a Mann-Whitney test was then used.
Is there an estimate of variation within each group of data?	The Brown-Forsythe test was used to assess variance among groups analyzed.
Is the variance similar between the groups that are being statistically compared?	If variances differed significantly, the non-parametric Kruskal-Wallis test with post-hoc Dunn's multiple comparison was used to confirm statistical significance between groups. For two groups a Mann-Whitney test was then used.

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 of all antibodies used in this study are listed in the materials and methods section
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 lline used in this study is the HEK293T cell line purchased from ATCC.

* 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.	These details are specified in the materials and methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	This statement is included in the materials and methods section.
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.	We hereby confirm the compliance to ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	
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.	
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	
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.	
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.	

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	
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).	
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).	
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	
22. 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.	

G- Dual use research of concern

23. 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.	