THE
EMBO
JOURNAL

Manuscript EMBO-2016-95148

VCP/p97 cooperates with YOD1, UBXD1 and PLAA to drive clearance of ruptured lysosomes by autophagy

Chrisovalantis Papadopoulos, Philipp Kirchner, Monika Bug, Daniel Grum, Lisa Koerver, Nina Schulze, Robert Poehler, Alina Dressler, Sven Fengler, Khalid Arhzaouy, Vanda Lux, Michael Ehrmann, Conrad C. Weihl, Hemmo Meyer

Corresponding author: Hemmo Meyer, Universitat Duisburg-Essen

Review timeline:

Submission date:	29 June 2016
Editorial Decision:	13 July 2016
Revision received:	31 August 2016
Editorial Decision:	07 September 2016
Revision received:	15 September 2016
Accepted:	17 September 2016

Editor: Andrea Leibfried

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 13 July 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. As your manuscript had been under consideration elsewhere before, it has now been seen by two arbitrating referees, who had access to the initial concerns raised as well as to your point-by-point response to them. I enclose the comments of these referees on the current version of your manuscript below. As you will see, the arbitrating referees find your manuscript interesting and endorse publication of a revised version in The EMBO Journal. Some concerns remain that should be addressed as outlined by referee #2: The interaction data should be recapitulated in the context of LLOMe treatment and some inconsistencies regarding the sequence of events during lysophagy need further attention. Furthermore, co-localization data are requested as well as analysis of autophagic flux in the context of p97/cofactor knockdown. Finally, please quantify all data and add statistical analyses.

I would thus like to invite you to submit a revised version of your work addressing the points mentioned above.

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

Please note that I will not include the previous reports nor your previous point-by-point response in this file.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision. Please let me know in case you would like to discuss individual points further.

REFEREE REPORTS

Referee #1:

The manuscript reports a novel complex that is involved in lysosome autophagy. The complex is composed of p97, UBXD1, PLAA and the DUB, YOD1, which was termed ELDR. The pathway specifically targets K48-linked ubiquitin conjugates on damaged lysosomes to promote autophagosome formation. They aslo argue that only a subpopulation of damaged lysosomes is conjugated
with K48 chains, as well as that some autophagosomes form early without involvement of K48 chains and independently of the EDLR components. This is a very exciting and novel finding as there are might be different pathways of degrading lysosome based on different signals of autophagy receptors, which is similar to distinct xenophagy pathways. The biochemical and cellular assays are provided to support the requirement of removal of K48 chains from lysosomal surface as a prerequisite for efficient removal of damaged lysosomes by autophagy. In particular the authors show that K48-conjugates need to be removed by p97 with help of the ELDR factors for autophagosome formation. YOD1 specifically cleaves K48 chains and overexpression of YOD1-CS inhibits removal of K48-conjugates on damaged lysosomes and reduces association of LC3-positive membranes. The authors also study in details the ELDR complex formation. They show that ubiquitin-binding by YOD1 stimulates interaction of YOD1 with p97 along with UBXD1 and PLAA and, in turn, p97 activates ubiquitin binding of YOD1. The stoichiometry of these complexes appear to be regulated dynamically within the cell.
Overall this is a complete study identifying a novel pathway for the removal of a subpopulation of lysosomes.

Referee #2:

Although the role of p97 is well characterized in the ERAD pathway, Papadopoulos et al. now showed a novel role of p97 in lysophagy. They showed that p92 works together with YOD1, UBXD1 and PLAA cofactors to facilitate the removal of damaged lysosomes via autophagy (lysophagy). Papadopoulos et al. also showed the dynamics of K63 and K48 ubiquitination during the course of lysosomal recovery, and based on their co-localization study and functional analysis, authors proposed that K48 has to be removed by p97 and its cofactors for lysophagy. Although their work lacks insights into why K48 removal is needed to lysophagy, it is still potentially interesting and also very informative for this field. However, I feel that several critical points have to be solved before the paper can be published in the EMBO journal. Upon the special request from the editor, I summarize my main concerns in light of the comments from the previous round of peer-review elsewhere:

1. The additional experiments in Fig. 3D-F support the authors' conclusion. However, the authors only showed the transient interaction in the normal condition. Even though the interaction is transient, they should check and show if this interaction is enhanced upon LLOMe using their substrate trapping mutants and by co-IP experiments. The previous reviewer 3 also raised similar points. This is critical since p97 and its cofactors are recruited to damaged lysosomes.

2. The experiments in Fig. 7D led to further inconsistencies. Specifically, in Figure S6A the recruitment of LC3 precedes the GFP-p97 localization. This is clearly opposite to the result from Fig.7D, which shows that YOD is recruited to latex beads prior to LC3. If p97 mediated K48 removal is critical for autophagy, how do the authors explain this observation? If the author's hypothesis is true, p97 and its cofactor have to be recruited in advance to LC3. The authors should clarify this point.

3. Some of the data are still not convincing because of the lack of quantification and statistics, for instance in Fig. S6 B and C. The authors should show statistics to convince the reader that K48 is really affected by knockdown and that the co-localization is enhanced by overexpression of cofactors.

4. The authors should directly show that p97 and cofactors are recruited to Gal3 positive dots on lysosomes, which can be achieved by triple staining with anti-Gal3, Lamp1 and p97. This critical data is missing.

5. The functional experiment in Fig. 7D itself is informative. However, as I mentioned above, the authors should clarify the inconsistent results within the paper between Fig. S6A and Fig.7C. In addition, the authors should check if LC3 lipidation and flux are also affected by knockdown of p97 and its cofactors. This is a critical experiment.

1st Revision - authors' response 31 August 2016

We thank the referees for the positive and thoughtful reviews. We believe we have addressed all concerns. In particular, we have provided additional quantification and statistical analysis. Moreover, we added requested colocalisation data and biochemical analysis of LC3 lipidation and turnover. In addition, we clarified the apparent inconsistency in the model with respect to the timing of LC3 recruitment and adapted the text to make this clearer for the reader. Lastly, with respect to the request for further co-IP experiments, we highlighted the specific properties of p97 cofactor interactions and how that affects the biochemical analysis of stress-induced complex assembly.

Referee #1:

The manuscript reports a novel complex that is involved in lysosome autophagy. The complex is composed of p97, UBXD1, PLAA and the DUB, YOD1, which was termed ELDR. The pathway specifically targets K48-linked ubiquitin conjugates on damaged lysosomes to promote autophagosome formation. They aslo argue that only a subpopulation of damaged lysosomes is conjugated
with K48 chains, as well as that some autophagosomes form early without involvement of K48 chains and independently of the EDLR components. This is a very exciting and novel finding as there are might be different pathways of degrading lysosome based on different signals of autophagy receptors, which is similar to distinct xenophagy pathways. The biochemical and cellular assays are provided to support the requirement of removal of K48 chains from lysosomal surface as a prerequisite for efficient removal of damaged lysosomes by autophagy. In particular the authors show that K48-conjugates need to be removed by p97 with help of the ELDR factors for autophagosome formation. YOD1 specifically cleaves K48 chains and overexpression of YOD1-CS inhibits removal of K48-conjugates on damaged lysosomes and reduces association of LC3-positive membranes. The authors also study in details the ELDR complex formation. They show that ubiquitin-binding by YOD1 stimulates interaction of YOD1 with p97 along with UBXD1 and PLAA and, in turn, p97 activates ubiquitin binding of YOD1. The stoichiometry of these complexes appear to be regulated dynamically within the cell.
Overall this is a complete study identifying a novel pathway for the removal of a subpopulation of lysosomes.

Referee #2:

Although the role of p97 is well characterized in the ERAD pathway, Papadopoulos et al. now showed a novel role of p97 in lysophagy. They showed that p92 works together with YOD1, UBXD1 and PLAA cofactors to facilitate the removal of damaged lysosomes via autophagy (lysophagy). Papadopoulos et al. also showed the dynamics of K63 and K48 ubiquitination during the course of lysosomal recovery, and based on their co-localization study and functional analysis, authors proposed that K48 has to be removed by p97 and its cofactors for lysophagy. Although their work lacks insights into why K48 removal is needed to lysophagy, it is still potentially interesting and also very informative for this field. However, I feel that several critical points have to be solved before the paper can be published in the EMBO journal. Upon the special request from the editor, I summarize my main concerns in light of the comments from the previous round of peer-review elsewhere.

1. The additional experiments in Fig. 3D-F support the authors' conclusion. However, the authors only showed the transient interaction in the normal condition. Even though the interaction is transient, they should check and show if this interaction is enhanced upon LLOMe using their substrate trapping mutants and by co-IP experiments. The previous reviewer 3 also raised similar points. This is critical since p97 and its cofactors are recruited to damaged lysosomes.

On this point, we respectfully disagree with the reviewer because of the particular properties of the p97 system that makes it impossible to isolate stress-induced cofactor complexes. The Deshaies laboratory recently demonstrated that p97-cofactor interactions are very dynamic and that isolated complexes disassemble rapidly within minutes after lysis (Xue et al., Mol Cell Proteomics. 2016 Jul 12. pii: mcp.M116.061036. [Epub ahead of print]). This is consistent with our data. We conducted the experiment as requested by the reviewer. We do see strong stimulation of ELDR complex assembly when we IP the YOD1-CS from the p97-EQ background compared to YOD1-wt (Figure 3E), showing that they all can bind in a substrate-dependent manner. However, there is no further stimulation if we treat with LLOMe most likely because binding is already maximal due to the mutants that are expressed for 24h (Figure for reviewer).

This does not diminish the significance of our findings. We demonstrate that all three cofactors (YOD1, UBXD1 and PLAA) are acutely recruited to the damaged lysosomes (now also shown by additional quantifications) and cooperate with p97 in driving lysophagy by regulating K48-conjugates on the damaged organelles. This is in contrast to the p47 cofactor that is not recruited to lysosomes and does not integrate into the ELDR complex. We believe that this demonstrates the specific damage-induced cooperation of the ELDR factors.

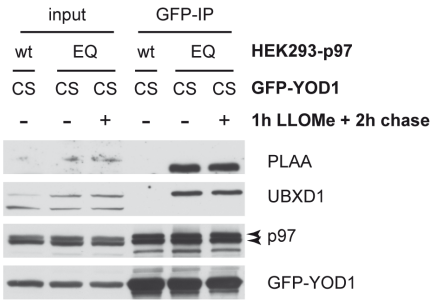

Figure for reviewer, related to Fig. 3E

Stable HEK293 cell lines were doxycycline-induced to express p97 wild-type (wt) or the ATPase-mutant E578Q (EQ) at near endogenous level, and transiently transfected with GFP-tagged YOD1-C160S catalytic mutant (CS). Cells were treated with LLOMe for 1 h or with vehicle and 2 h after washout, processed for co-immunoprecipitation with GFP nanobeads.

2. The experiments in Fig. 7D led to further inconsistencies. Specifically, in Figure S6A the recruitment of LC3 precedes the GFP-p97 localization. This is clearly opposite to the result from Fig.7D, which shows that YOD is recruited to latex beads prior to LC3. If p97 mediated K48 removal is critical for autophagy, how do the authors explain this observation? If the author's hypothesis is true, p97 and its cofactor have to be recruited in advance to LC3. The authors should clarify this point.

We are sorry if this point did not become clear in the text. The apparent inconsistency stems for the observation that there are two subpopulations with different timing and regulation of LC3 recruitment. The first population is only K63-modified and immediately recruits LC3-positive membranes, which explains the early appearance of LC3-positive autophagosomes.
The second population, although they are K63-modified, do not recruit LC3 immediately, but instead become modified with K48 chains, which interferes with LC3 recruitment as we demonstrate in Fig. 7. Only when p97 and the ELDR factors remove the K48 conjugates, can LC3 recruitment occur. This explains why on a global scale we observe LC3 association earlier than p97 recruitment in Fig. S6A, but on the individual lysosomes of the second population, LC3 is downstream of p97. This is now further supported by additional quantifications of p97/YOD1 on the two populations.
We had elaborated on the two populations in the discussion, but now have adapted the text and the abstract to make this point clearer.

3. Some of the data are still not convincing because of the lack of quantification and statistics, for instance in Fig. S6 B and C. The authors should show statistics to convince the reader that K48 is really affected by knockdown and that the co-localization is enhanced by overexpression of cofactors.

As requested, we have added the remaining quantification and statistics for Fig. S6B (effect on the persistence of K48 chains by siUBXD1 and siPLAA) and S6C (K48 is decreased by YOD1-wt and increased by YOD1-CS, while K63 is not affected). In addition, we included new quantification and statistics in Fig.1C (recruitment of endogenous p97 to vesicles upon LLOMe), Fig.1G (induction of Gal3-vesicles by tau), Fig. S2A now in Fig. 2B (Gal3 clearance in p97 mutant cells), Fig. S3B (cofactor recruitment to damaged lysosomes in Fig. 3C). Fig. S5D (localization of endogenous p97 to K48-positive lysosomes), Fig. S5E/F (colocalisation of K63 with UBXD1 and PLAA in Fig.S5E and comparison to K48 in Fig. 4F), Fig. S6A (time course of p97 recruitment). All quantifications support our original conclusions.

4. The authors should directly show that p97 and cofactors are recruited to Gal3 positive dots on lysosomes, which can be achieved by triple staining with anti-Gal3, Lamp1 and p97. This critical data is missing.

As requested, we have performed the triple localization (LAMP1, GFP-p97-EQ, mCherry-Gal3) and added in Fig. S5C. We provide the quantifications for p97 and also the cofactors in Fig. S3B. The data is in line with Figure S5A and B demonstrating that p97 and YOD1 localizes to LAMP1 compartments that are negative for Lysotracker and cathepsin-activity, hence to damaged lysosomes.

5. The functional experiment in Fig. 7D itself is informative. However, as I mentioned above, the authors should clarify the inconsistent results within the paper between Fig. S6A and Fig.7C. In addition, the authors should check if LC3 lipidation and flux are also affected by knockdown of p97 and its cofactors. This is a critical experiment.

We have clarified the apparent inconsistency between Fig. S6A and 7C in our comment above.

As requested, we have now included extensive new data addressing LC3 lipidation and flux in Western blot experiments. We depleted p97 and the ELDR cofactors and found that this leads to accumulation of LC3-II (Fig. S3C). Because the amount of LC3-II is not increased by BafA1 compared to the depletion control, we conclude that the depletions inhibit autophagic flux, consistent with our model. We repeated the experiment in depleted cells treated with LLOMe and followed the clearance of LC3-II. Again, depletions led to persistence of LC3-II 10 h after LLOMe in the ELDR factor depletions (Figure S3D). Interesting, depletion of another p97 cofactor, p47, which we found was not directly involved in lysophagy because it was not recruited to the damaged lysosomes, did not increase LC3-II level and is therefore a good control. p47 even slightly reduced LC3-II formation consistent with a role upstream in LC3 activation and in line with previous results (Zhang et al., PNAS 2015 Apr 7;112(14):E1705-14).

In addition, we have analyzed LC3-II formation and clearance in U2OS cells that express p97 wild-type or disease mutants. LC3-II levels are not significantly increased by expression of the mutants in untreated cells, but they increased and persisted after induction with LLOMe in the mutants compared to the controls (Fig. 2C and S2B). This demonstrates that the flux in the autophagic clearance of lysosomes is inhibited rather than LC3-II activation. Again, the result supports our conclusions.

2nd Editorial Decision 07 September 2016

Thank you for submitting your revised manuscript for our consideration. Your manuscript has now been seen once more by referee #2 (see comments below), and I am happy to inform you that the referee now supports publication, pending satisfactory minor revision. I would therefore like to ask you to address referee #2's suggestion and to provide a final version of your manuscript.

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we should have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript! to request original versions of figures and the original images that were used to assemble the figure.

REFEREE REPORT

Referee #2:
Most of my concerns have been adequately addressed. Minor point: Page 6, line 9 from the bottom. "indicating impairment in autophagosome formation" should be "suggesting impairment in autophagy flux"

2nd Revision - authors' response 15 September 2016

REFEREE REPORT

Referee #2:
Most of my concerns have been adequately addressed. Minor point: Page 6, line 9 from the bottom. "indicating impairment in autophagosome formation" should be "suggesting impairment in autophagy flux."

We thank the referee for the positive review. We changed the text as suggested (page 6, line 10 from bottom).

3rd Editorial Decision 17 September 2016

Thank you for submitting your revised manuscript to us. I appreciate the introduced changes and I am happy to accept your manuscript for publication in The EMBO Journal.
Congratulations!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Hemmo Meyer
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2016-95148

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).

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?	We described the chosen sample size for all data in figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
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.	NA
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?	The number of independent experiments performed, the type of statistical tests and if SD or SEM is shown are specified in the figure legends for every figure.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All column plots, graphs and statistical analysis wer done using GraphPad Prism. We used student's t-test when the distribution was normal. Otherwise we used a non-parametric test (Mann-Whitney U) .
Is there an estimate of variation within each group of data?	We show standard deviation or standard error of the mean as described in the figure legend.
Is the variance similar between the groups that are being statistically compared?	Statistical analysis was done with GraphPad prism. The software calculates the homogenity of variances by means of the F-test in case of the student's t-test. Was the F-test for homogenity of variances significant , we used a non-parametric test.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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-tun

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/

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).	Antibodies used are described in Appendix Material and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We described the source of cell lines. All cell lines used in this study were tested 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	The use of patient biopsy material was approved by Washington University School of Medicine IRB.
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.	Patient material was obtained for clinical /diagnostic purposes. No material was obtained for research purposes. The utilization of existing biopsy material is approved under local IRB.
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.	Patient material is a limited resource and is subject to limitations of ist use.
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 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	NA
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).	NA
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. 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	NA
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.	NA

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.	No