EMBO Molecular Medicine

Control of Anterior GRadient 2 (AGR2) dimerization links endoplasmic reticulum proteostasis to inflammation

Marion Maurel, Joanna Obacz, Tony Avril, Yong-Ping Ding, Olga Papadodima, Xavier Treton, Fanny Danie, Eleftherios Pilalis, Johanna Hörberg, Wenyang Hou, Marie-Claude Beauchamp, Julien Tourneur-Marsille, Dominique Cazals-Hatem, Lucia Sommerova, Afshin Samali, Jan Tavernier, Roman Hrstka, Aurélien Dupont, Delphine Fessart, Frédéric Delom, Martin E. Fernandez-Zapico, Gregor Jansen, Leif A. Eriksson, David Y Thomas, Loydie Jerome-Majewska, Ted Hupp, Aristotelis Chatziioannou, Eric Chevet, Eric Ogier-Denis

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Editor: Céline Carret

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 8 January 2019

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript.

You will see from the comments pasted below that both referees are supportive of publication, only suggesting limited additions. I'd like to insist on referee 2's comment to identify the AGR2 secretory pathway in order to deepen the putatively targetable therapeutic signaling axes.

We would therefore welcome the submission of a revised version within three months for further consideration and would like to encourage you to address all the criticisms raised as suggested to improve conclusiveness and clarity. Please note that EMBO Molecular Medicine strongly supports a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

This manuscript deals with the anterior gradient 2 (AGR2), a folding catalyst/chaperone that resides in the endoplasmic reticulum (ER) and has been implicated in ER proteostasis and associated with intestinal inflammation. The study provides molecular insights into the AGR2 mode of action with an emphasis on the regulation of AGR2 assembly into homodimers and also addresses the involvement of AGR2 and its dimers in inflammatory bowel disease (IBD) and Crohn's disease.

Major findings:

1. AGR2 forms stress-regulated homodimers in the ER:
• Molecular dynamics confirmed AGR2 homodimerization via E60 and verified the reduced dimer stability of the E60A mutant.
• AGR2 dimeric / monomeric equilibrium was investigated by molecular modeling.
• Chemical cross-linking combined with non-reducing and reducing electrophoresis followed by immunoblotting showed the predominance of AGR2 homodimers.
• The ERMIT assay, in which IRE1 luminal domain was replaced with various AGR2 constructs (wild-type, E60A, C81A, E60A/C81A double mutant), verified AGR2 homodimerization in the ER, while E60 key role in the homodimers formation was deduced from the dimerization defective mutants (E60A, E60A/C81A).
• Upon ER stress (induced by DTT, thapsigargin or tunicamycin), AGR2 homodimers dissociated in a dose-dependent manner, and 35S-methionine pulse-chase followed by AGR2 immunoprecipitation revealed ER stress-induced altered association of AGR2 with nascent proteins.

2. Identification AGR2 interactors as modulators of AGR2 homodimerization and characterization of TMED2 as a major enhancer of AGR2 dimerization:
• A specific ERMIT-based siRNA screen identified inhibitors and enhancers of AGR2 homodimerization. Their functional pathways implicated the dimeric AGR2 in protein folding and the monomeric form in managing ER stress.
• TMED2 was identified as a positive regulator that enhanced AGR2 homodimerization and co-immunoprecipitation revealed a TMED2-AGR2 complex that dissociated upon ER stress.
• AGR2 AA (K66A/Y111A), a mutant unable to interact with TMED2 (generated according to molecular modeling), failed to co-immunoprecipitate with TMED2.
• Protein-protein docking showed that TMED2 interaction with AGR2 monomers was unstable, while its interaction with AGR2 dimers rendered several conformers in which TMED2 simultaneously interacted with both AGR2 monomers.
• TMED2 overexpression resulted in enhanced AGR2 homodimerization, yet led to reduced AGR2 expression and reduced ERMIT signals due to AGR2 dwindling.
• TMED2 silencing led to enhanced AGR2 expression but decreased ERMIT signals, reflecting reduced homodimerization.

3. AGR2 dimerization had no effect on its chaperone activity but AGR2-TMED2 interplay regulated protein folding and trafficking and contributed to ER protein quality control:
• AGR2 regulation of cargo secretion was followed by ERMIT, monitoring the interaction of the constitutively monomeric AGR2 E60A with two plasma-membrane GPI-anchored proteins, CD59 and LYPD3.
• AGR2 contribution to the ER quality control and to protein secretion was followed with CD59 and its misfolded ER-retained C94S mutant, and both CD59 and C94S interacted similarly with either AGR2 or the TMED2 interaction-defective AGR2 AA.
• AGR2 silencing led to reduced expression of intracellular CD59 and C94S with no effect on their cell surface expression, suggesting a role of intracellular AGR2 in ER quality control.
• TMED2 silencing led to reduced expression of intracellular CD59 and C94S accompanied by similar effect on their cell surface expression.
• Overexpression of either AGR2 or AGR2 AA restored the total and cell surface expression of CD59 and C94S.
• While AGR2 silencing did not affect the secretion of alpha-1-antitrypsin under basal conditions, the ER retention of alpha-1-antitrypsin decreased in the absence of AGR2 upon ER stress.

• A role for AGR2 in ER proteostasis was deduced from the stabilized expression of MUC2 in the presence of AGR2.

4. Overexpression of TMED2 decreased AGR2 levels not via ERAD but through autophagy:
• Pharmacological inhibitors of the ERAD pathway did not recover the decreased levels of intracellular AGR2 induced by TMED2 overexpression.
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5. Pathophysiological relevance of AGR2 and its dimerization to intestinal inflammation in mice models and patients biopsies:
• In mice, alteration of TMED2 expression was related to AGR2-mediated monocytes attraction.
• Expression levels of AGR2, TMED2 and CD163 were altered in biopsies from IBD patients when compared to healthy controls.

This study is a nice example of the emerging link between ER proteostasis and the pathophysiology of various diseases. Proper folding of newly synthesized proteins in the ER is assisted by molecular chaperones and folding catalysts, whereas ER quality control and degradation mechanisms handle the burden of misfolded proteins. In this manuscript the authors address molecular mechanisms underlying the involvement of AGR2 in regulating ER protein quality control and the development of IBD. The major focus of this study is AGR2 homodimers: their regulation by assembly modulators, their response to ER stress and their effect on AGR2 function. To unveil AGR2 molecular functions and pathophysiological roles, the authors employed several complementary approaches with carefully designed controls, to provide clear and comprehensive data and straightforward conclusions. Most impressive is the development of ERMIT, an ER mammalian protein-protein interaction trap, which allowed them to detect AGR2 interactors. Using this elegant protein-protein interaction screen, the authors identified TMED2 as a major and specific enhancer of AGR2 dimerization and discovered that AGR2 homodimers were disrupted upon ER stress. They also demonstrated that variations in TMED2 expression resulted in AGR2 secretion and pro-inflammatory phenotypes, correlating deregulated levels of AGR2 dimerization modulators with IBD and Crohn's disease severity. The authors raise the possibility that AGR2 dimers act as sensors of ER homeostasis, which are disrupted upon ER stress and promote the secretion of AGR2 monomers, possibly representing systemic alarm signals for pro-inflammatory responses.

Minor points:
1. Indeed, in Fig. 3E, the levels of AGR2 co-immunoprecipitated with TMED2 are higher for the wild-type AGR2 (left panel) than for the TMED2 interaction-defective AGR2 AA mutant (right panel). However, in the input lower panels, wild-type AGR2 is hardly detected and no AGR2 AA is detected whatsoever. It confirms that TMED2 overexpression leads to decreased levels of AGR2, although independently of TMED2-AGR2 interaction (Fig. 3E,F), yet one wonders what is the "source" of the co-immunoprecipitated AGR2 species.

Referee #2 (Comments on Novelty/Model System for Author):

AGR2 perturbation in mammals influences disease processes including cancer progression and drug resistance, asthma, and inflammatory bowel disease. This article is a tour-de-force with data ranging from structural biology, biochemistry, proteomics, electron microscopy, mouse genetics to clinics to demonstrate the molecular mechanism of how AGR2 may participate to inflammation.

The novel results are that 1/modulation of AGR2 dimer formation by point mutations and expression of partner TMED2 (identified in this study) expression induces pro-inflammatory

phenotypes via autophagy-dependent processes or secretion of AGR2 and 2/the expression levels of AGR2 dimerization modulators including TMED2 are selectively deregulated in Crohn's disease.

The methods used are adequate and the results were well acquired and analysed and fairly discussed. The biochemical and cell biological data are particularly convincing.

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The one missing piece of data which would clarify the process described and potentially develop therapeutic strategy concerns the secretory pathway of AGR2: is it a conventional secretion ER-Golgi-PM or unconventional one from ER to PM or does it involve endosomes?

1st Revision - authors' response 1 March 2019

REVIEWER 1

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We thank this reviewer for this very clear description of the main findings presented in our work

This study is a nice example of the emerging link between ER proteostasis and the pathophysiology of various diseases. Proper folding of newly synthesized proteins in the ER is assisted by molecular chaperones and folding catalysts, whereas ER quality control and degradation mechanisms handle the burden of misfolded proteins. In this manuscript the authors address molecular mechanisms underlying the involvement of AGR2 in regulating ER protein quality control and the development of IBD. The major focus of this study is AGR2 homodimers: their regulation by assembly modulators, their response to ER stress and their effect on AGR2 function. To unveil AGR2 molecular functions and pathophysiological roles, the authors employed several complementary approaches with carefully designed controls, to provide clear and comprehensive data and straightforward conclusions. Most impressive is the development of ERMIT, an ER mammalian protein-protein interaction trap, which allowed them to detect AGR2 interactors. Using this elegant protein-protein interaction screen, the authors identified TMED2 as a major and specific enhancer of AGR2 dimerization and discovered that AGR2 homodimers were disrupted upon ER stress. They also demonstrated that variations in TMED2 expression resulted in AGR2 secretion and pro-inflammatory phenotypes, correlating deregulated levels of AGR2 dimerization modulators with IBD and Crohn's disease severity. The authors raise the possibility that AGR2 dimers act as sensors of ER homeostasis, which are disrupted upon ER stress and promote the secretion of AGR2 monomers, possibly representing systemic alarm signals for pro-inflammatory responses.

We thank this reviewer for the nice comments on our work

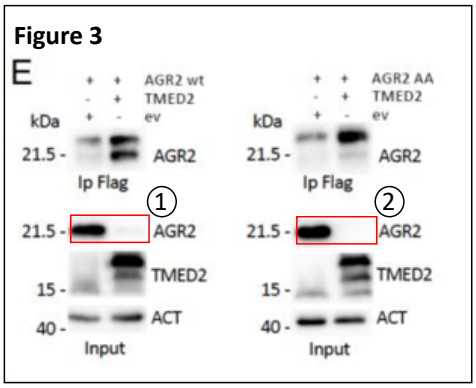
Reviewer#1 - remarks breakdown

Comment R1-1: Indeed, in Fig. 3E, the levels of AGR2 co-immunoprecipitated with TMED2 are higher for the wild-type AGR2 (left panel) than for the TMED2 interaction-defective AGR2 AA mutant (right panel). However, in the input lower panels, wild-type AGR2 is hardly detected and no AGR2 AA is detected whatsoever. It confirms that TMED2 overexpression leads to decreased levels of AGR2, although independently of TMED2-AGR2 interaction (Fig. 3E,F), yet one wonders what is the "source" of the co-immunoprecipitated AGR2 species.

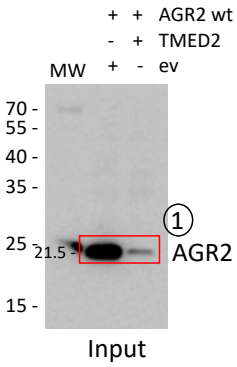
*Response R1-1: As indicated by reviewer#1, TMED2 overexpression leads to decrease AGR2_wt expression levels (**Figure 3E** provided in **Figure R1A**, **Figure 3F**, and **Figure R1B**). We observed also a decreased expression of AGR2_AA upon TMED2 overexpression (**Figure R1A** and **Figure R1C**) although its AGR2 mutant should not be able to interact with TMED2 according to our in silico structural analyses of protein/protein interactions. Over-exposed images of **Figure 3E** discussed by the reviewer#1 are shown in **Figure R1B** and **R1C**, and confirm the presence of reduced amounts of AGR2_wt and AGR2_AA in TMED2 over-expressing cells. The source of AGR2 observed in co-immunoprecipitation experiments is therefore provided by the cells. As suggested by the reviewer#1, this underlines the possible that TMED2 regulates AGR2 expression independently to direct TMED2/AGR2 interactions. This might occur through mechanisms associated with some unconventional release of AGR2 in the extracellular space (see below). A sentence has been added to address this point on p9 of the revised manuscript.*

Figure R#1_1

R#1_1A



R#1_1B



R#1_1C

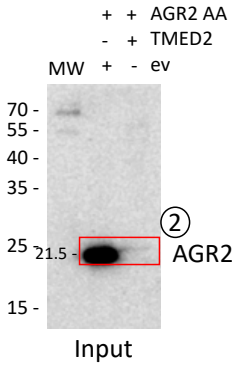


***Figure R#1-1: Validation of AGR2/TMED2 interaction.** Co-immunoprecipitation of AGR2 wild-type (wt, left panel) or AGR2 AA mutant (right panel) with TMED2 in HEK293T cells. Immunoprecipitation was performed with mouse anti-Flag antibody to pull-down the ectopic protein tag **(R#1_1A)**. Longer exposure of the western blots framed in red boxes (1) and (2) were provided in **R#1_1B** and **R#1_1C** respectively.*

REVIEWER 2 (Comments on Novelty/Model System for Author):

AGR2 perturbation in mammals influences disease processes including cancer progression and drug resistance, asthma, and inflammatory bowel disease. This article is a tour-de-force with data ranging from structural biology, biochemistry, proteomics, electron microscopy, mouse genetics to clinics to demonstrate the molecular mechanism of how AGR2 may participate to inflammation.

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We thank this reviewer for this very kind comments on our work.

Reviewer#2 - remarks breakdown
Comment R2-1: The one missing piece of data which would clarify the process described and potentially develop therapeutic strategy concerns the secretory pathway of AGR2: is it a conventional secretion ER-Golgi-PM or unconventional one from ER to PM or does it involve endosomes?

Response R2-1: We thank the reviewer#2 for this important remark. To test if AGR2 secretion is dependent of the endo-lysosomal pathway, we have performed additional experiments in which AGR2 was overexpressed in HEK293T cells which were treated or not with brefeldin A (BFA) and/or bafilomycin A. BFA inhibits secretory and transmembrane protein transport from the ER to the Golgi apparatus by blocking a membrane-associated ARF-GEF activity (sec7 domain containing proteins) and bafilomycin A is an inhibitor of the endosomal V-ATPase and prevents the entry of protons in the endosome, hence its acidification. Total and secreted AGR2 levels were analysed by western blot.

Figure R#2_1

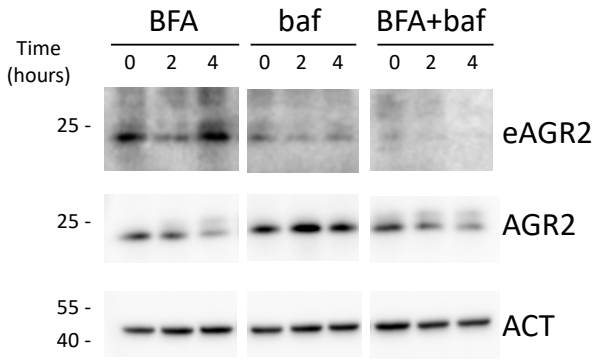

Figure R#2-1: Cellular mechanisms of AGR2 secretion. *HEK293T cells were transfected with AGR2_wt and were treated with brefeldin A (BFA, 5 µg/ml), bafilomycin A1 (baf, 200 nM), or both (B+B). Western blot analysis of intracellular and secreted AGR2 upon 2 and 4 hours treatment were shown. eAGR2, extracellular AGR2; AGR2, intracellular AGR2. Actin (ACT) served as a loading control.*

As shown in Figure R#2_1, treatments with drugs were carried out for relatively short time periods to minimize the impact on the ER stress response. BFA treatment led to reduced intracellular AGR2 expression as well as the appearance of a lower mobility form that might correspond to absence of peptide signal cleavage as previously shown (Genes Chromosomes Cancer. 2005 Jul;43(3):249-59) which was observed after 2 to 4 hours of treatment. In contrast, no change was observed upon treatment with bafilomycin. As expected BFA treatment reduced AGR2 secretion after 2 hours, but led to further release of AGR2 after 4h, suggesting a bypass of the conventional secretory pathway. Again bafilomycin had not effect on AGR2 secretion. Interestingly the secretion of AGR2 was abrogated upon treatment with both BFA and bafilomycin thus suggesting that the secretion of AGR2 might be in part dependent on the endo-lysosomal system as also observed upon overexpression of TMED2 in Fig 5 of the manuscript. We have added this figure in Supplemental material (Fig S5D) and a corresponding sentence on p12 of the revised manuscript.

2nd Editorial Decision 22 March 2019

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending minor editorial amendments.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

The authors have adequately responded to the comments raised by both reviewers and revised the manuscript accordingly.

Referee #2 (Remarks for Author):

The authors have satisfactorily answered the reviewers' requests.

2nd Revision - authors' response 28 March 2019

Authors made the requested editorial changes.

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: Eric Chevet
Journal Submitted to: EMBO MOLECULAR MEDICINE
Manuscript Number: EMM-2018-10120

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	By default all experiments carried out are with a minimum of n=3
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For colonic Ligation loop assays, n=3 for tunicamycin treated and n=3 for vehicle treated animals. For TMED2 mutant mice, immunohistochemical analysis of colon and ileum tisssues from untreated wt (n=6) and TMED2 hypomorph mice (n=6).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusion performed
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.	No treatment in animal experiments
For animal studies, include a statement about randomization even if no randomization was used.	Random selection of male mice in both wt and TMED2 groups.
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.	For all experiments requiring
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding performed
5. For every figure, are statistical tests justified as appropriate?	YES
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	YES, included in the GrapPad software Prism
Is there an estimate of variation within each group of data?	YES, included in the GrapPad software Prism
Is the variance similar between the groups that are being statistically compared?	YES, included in the GrapPad software Prism

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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http://www.consort-statement.org/checklists/view/32-consort/66-title

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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).	This is provided 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.	This is provided in the materials and methods section

* 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.	Bred in-house 2 months old male C57BL/6 mice were used (for ligated-colonic loops assays). For TMED2 mutant mice, the 99J (TMED2 mutant) mouse line was generated on a C57/BL6J genetic background and maintained on a mixed C3H genetic background (C3HeB/FeJ and C3HeB/FeV) as previously described (Hou et al., 2017). Six months old male TMED2 mutants (n = 6) and their wild-type littermate (n = 6) were analyzed.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All the in vivo experiments (ligated-colonic loop assays) were approved by and conducted in accordance with the ethical guidelines of the Local Animal Experimentation Committee (Xavier Bichat Medical School) and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Alternatively, procedures and experiments carried out regarding TMED2 mutant animals were performed according to the guidelines of the Canadian Council on Animal Care and approved by the Animal Care Committee of the Montreal Children's Hospital. In both cases efforts were made to minimize any pain or discomfort , and the minimum number of animals was used.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	All human samples used for the analyses shown in this manuscript were provided by the Centre de Ressources Biologiques from the Beaujon/Bichat Hospitals under the auspices of French National autorities.
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.	ok
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
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).	N/A
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).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NO