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A SNARE geranylgeranyltransferase essential for the organization of the Golgi apparatus

Ryutaro Shirakawa, Sakurako Goto-Ito, Kota Goto, Shonosuke Wakayama, Haremaru Kubo, Natsumi Sakata, Duc Anh Trinh, Atsushi Yamagata, Yusuke Sato, Hiroshi Masumoto, Jinglei Cheng, Toyoshi Fujimoto, Shuya Fukai, and Hisanori Horiuchi

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1st Editorial Decision 20th December 2019

Thank you for submitting your manuscript entitled "A SNARE geranylgeranyltransferase essential for the organization of the Golgi apparatus" [EMBOJ-2019-104120] to The EMBO Journal. Your study has been sent to three referees for evaluation, whose reviews are enclosed below.

As you can see, the referees highly appreciate your study and support its publication in The EMBO Journal without additional experiments. However, they request you to expand the discussion on the related manuscript recently published by Kuchay et al., 2019.

Given the referees' positive evaluation, I would like to invite you to revise the manuscript in response to the referee reports.

REFEREE REPORTS

Referee #1:

This is an excellent, very thorough piece of work describing the discovery and characterization of a new prenyl-transferase. The work shows that PTAR1, a protein with homology to the two known prenyl-transferase alpha subunits (FNTA and RabGGTA), is indeed an alpha subunit that pairs with the known beta subunit RabGGTB for the geranylgeranylation of at least one substrate protein, the SNARE YKT6. This work overlaps a paper from Kuchay et al. published June 17, 2019 in Nature Structural & Molecular Biology, which also reported that PTAR1 pairs with RabGGTB to effect geranylgeranylation. The present manuscript from Shirakawa et al. appears to have been done contemporaneously and independently and as discussed below, the Shirakawa paper goes further than the Kuchay paper in providing new insights.

Obviously, there is considerable overlap between the two papers. For instance, both papers show that PTAR1 heterodimerizes with the RabGGTase beta subunit to form a new GGTase (GGTase-III), an enzyme that mediates geranylgeranylation. In both cases, this is shown unequivocally biochemically and by x-ray structure. However, the Shirakawa et al. paper goes substantially further than the Kuchay paper in providing a number of important new insights.

My main issue is that, while the Kuchay paper will likely predate publication of the Shirakawa paper by at least 9 months or so, the Kuchay paper is not well discussed in the current manuscript, being mentioned only in the penultimate paragraph of the Discussion. Furthermore, while there is significant overlap between the two papers, there also is substantial conflict regarding the proposed prenylation mechanisms (discussed below). Indeed, SFig. 7 of Shirakawa et al. appears to fully refute a key finding of the Kuchay paper regarding FBXL2 as a GGTase-III substrate. The authors need to expand upon this in their discussion.

Overall, I find the Shirakawa paper to a beautiful, well-written and very thorough piece of work. It covers a lot of ground and provides a number of important new insights. My one criticism that I voice above is that the authors need to better discuss the points of overlap as well as substantial differences with the Kuchay et al. paper. The points below highlight new insights that are uniquely provided by the Shirakawa paper. Otherwise, I have no criticisms or experimental suggestions.

1. The two papers each identify a new single substrate for the new PTAR1/RabGGTB geranylgeranyl-transferase. Kuchay identify FXBL2, an F-box protein subunit of a membrane-localized ubiquitin ligase complex, while Shirakawa identifies the YKT6 SNARE protein. The YKT6 story is quite detailed and very thoroughly documented, while the Kuchay et al. FBXL2 story, seems frankly, a bit confusing.

Both substrate proteins end with the familiar -CaaX tetrapeptide prenylation consensus, however both also have additional proximal cysteines that likely also are used for lipidation, either additional prenylation or possibly palmitoylation.

The YKT6 C-terminal sequence is -CCAIM. Here, the long-standing story on YKT6 lipidation has been that the cysteine of the -CaaX consensus gets farnesylated by FTase, while the adjacent upstream cysteine is palmitoylated in regulated fashion (like N- or H-Ras). The regulated palmitoylation was thought to regulate localization, with prenylated, but unpalmitoylated YKT6 adopting a "closed" conformation, which localized largely to the cytoplasm and with the dually lipidated form acting at the membrane as a SNARE. This was a nice model that nicely fitted with what was known at the time about protein lipidation.

The Shirakawa paper now revises this model. The -CaaX cysteine is still farnesylated by FTase. However, the new view has the adjacent cysteine now being geranylgeranylated by the new GGTase-III. The authors provide unequivocal support for this model, both biochemically, using mutated YKT6s, mutated individually for the two cysteines and importantly, via their crystallography which identifies two hydrophobic tunnels adjacent to the active site of the RabGGTB subunit. One tunnel accommodates the YKT6 farnesylated cysteine (farnesylation appears to normally occur as a first step), while the second tunnel likely accommodates both the GG-PP substrate initially and then the geranylgeranlated cysteine following the addition. The authors go on to show that this dual tunnel feature is unique to RabGGTB (among the three different prenyl transferase beta subunits). The authors further discuss that this dual tunnel feature is likely used in RabGGTB's other role as a general Rab protein prenyl transferase, where two adjacent cysteines are again dually prenylated.

The prior model (i.e. farnesylation plus palmitoylation), the authors point out, was only weakly supported by data, with palmitoylation being detected under conditions employing different mutant YKT6s. Looking back at an old paper on the proteomic analysis of protein palmitoylation in yeast (the Roth et al., 2006 reference), I note that yeast YKT6 was the one protein that was anticipated to be palmitoylated that was conspicuously missing from the purified palmitoyl-proteome. Roth et al. attributed this absence to likely low fractional palmitoylation of YKT6. However, the new Shirakawa et al. results provide a better explanation.

1a. The Kuchay story on FBXL2 geranylgeranylation is a bit more confusing. The FBXL2 C-

terminus is -CRCCVIL. The cysteine of the -CaaX tetrapeptide would seem to be a natural substrate of GGTase-I. However, the results provided by Kuchay are interpreted to support a model where this cysteine is primarily geranylgeranylated by the new GGTase-III. However, there remains some residual geranylgeranylation that is mediated by GGTase-I, which is particularly evident when GGTase-III is mutated. Thus, the two GGTases are suggested to act redundantly on the same cysteine, with GGTase-III being preferred. Disconcertingly however, SFig 7 of Shirakawa et al. finds no change in PTAR1's plasma membrane localization in PTAR1-deficient cells. Furthermore direct assessment of geranylgeranylation yields results indicating FBXL2 to be exclusively modified by GGTase-I and not by GGTase-III.

The possibility of lipidation for the two other cysteines in the FBXL2 C-terminal sequence is not addressed in the Kuchay paper. A month ago, prior to reading Shirakawa et al., I would have predicted that these two cysteines would be palmitoylated (in analogy to H- or N-Ras). In light of the proposed Shirakawa mechanism, a better model might have the GGTase-III-mediated geranylation occurring on the cysteine immediately adjacent to the -CaaX cysteine, which would have been pre-modified by GGTase-I.

Bottomline, the important differences in the two papers, regarding proposed prenylation mechanisms (discussed above), needs to be better highlighted by the authors. Nonetheless, while I find the Shirakawa paper to be the better, more complete paper, the Kuchay paper still is quite significant, given its solid (and prior) documentation of PTAR1 as a GGTase-III subunit and it needs to be more clearly acknowledged earlier in the Shirakawa manuscript.

2. As with the Kuchay et al. paper, Shirakawa et al. report that they were initially unable to visual the substrate protein -CaaX C-terminus in co-crystals of substrate with GGTase-III, presumably due to excessive movement of the C-terminal peptide within their crystals. The key trick employed by Shirakawa that ultimately delivered visualization was to use a more native version of YKT6 substrate that was both pre-farnesylated and trimmed of its C-terminal tripeptide. The resulting visualization provides important new details about the GGTase-III prenylation, exposing the two hydrophobic tunnels, one of which gets filled with the farnesylated cysteine and the other, presumably with the GG-PP reactant. Another major take-away is that FTase-mediated farnesylation likely precedes the GGTase-III mediated geranylgeranylation of the adjacent cysteine.

3. Another plus for the Shirakawa paper, relative to Kuchay, is that provide a generalized characterization of the cellular defects imposed by PTAR1 knockout.

Referee #2:

It is rare that I get a paper that is as beautifully done as this one. Congratulations to the authors! This paper describes an entirely new class of prenyltransferase (named GGTase-III) that adds a geranylgeranyl group to the Golgi SNARE, Ykt6. The preferred substrate is a farnesylated Ykt6 and they show that the product in vitro and in cells is doubly modified. The authors include the structure of the product bound to the enzyme and functional studies in cells, showing under-glycosylation of LAMP protein and an altered Golgi complex structure by EM of knock out cells. The only question this reviewer has is whether there are any additional substrates for this enzyme. The authors could look in vitro at any cytosolic proteins that acquire biotin-precursor in reactions with and without the GGTase-III subunit, monitored by western blot with streptavidin. This is not essential for the present story but would be an interesting result. Overall, well done! -Suzanne Pfeffer (signed review).

Referee #3:

The manuscript „A SNARE geranylgeranyltransferase essential for the organization of the Golgi apparatus" by Shirakawa et al. reports the characterization of a putative prenyl transferase and the discovery of its target, the SNARE protein Ykt6.

Prenylation is an important post-translational modification occurring on the C-termini of specific proteins. So far, the enzymes Farnesyltransferase (FTase), Geranylgeranyltransferase I (GGTase I),

and GGTase II have been identified and characterized biochemically, structurally, and physiologically. In this manuscript, the authors report on the identification of a previously unrecognized GGTase that they term GGTase III. They were able to identify its target, the SNARE protein Ykt6. In contrast to other prenyltransferases, GGTase III can only prenylate Ykt6 after the processing of the target by 3 preceding post-translationally modifying enzymes: FTase, RCE, and ICMT. Hence, the C-terminus of Ykt6 gets farnesylated, proteolytically processed, and methylated before addition of the geranylgeranyl moiety by GGTase III.

The authors present a convincing study regarding the cellular function of GGTase III, its target identification, the structural features of the enzyme-target-complex, and the biochemical mechanism of geranylgeranyl transfer. I have enjoyed reading this work very much and I do not have any reservation concerning its publication. It will be interesting to see whether GGTase III may also have alternative targets. Previously it was thought that only small GTPases of the Rab family can be double prenylated (by GGTase II). However, this paper unexpectedly shows that also other and totally unrelated proteins (i.e. Ykt6) can be subject to double prenylation. This work may therefore form the basis for future studies on protein double prenylation. I would like to recommend publication of this paper enthusiastically.

Minor comments:
- Regarding the title of the manuscript: The title implies generality of SNARE prenylation. I would suggest being more specific since Ykt6 is the only discovered target SNARE for GGTase III.

- Please refrain from claims of novelty (e.g. "novel type of protein prenylation"). Prenylation in general and geranylgeranylation are known for many years. Also, the structure of GGTase III is homologous to other prenyl transferases. Thus, the finding is scientifically interesting, but not truly novel.

1st Revision - authors' response | 22nd January 2020

Response to the referees' comments

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3. Another plus for the Shirakawa paper, relative to Kuchay, is that provide a generalized characterization of the cellular defects imposed by PTAR1 knockout.

Response: We thank the reviewer for appreciating and summarizing our work, especially in relation to the preceding paper by Kuchay et al. We also thank the reviewer for the insightful suggestion that GGTase-III could geranylgeranylate the cysteines adjacent to the CAAX motif of FBXL2. According to the reviewer, we discussed the findings of Kuchay et al. in the Discussion and acknowledged the Kuchay paper in the Introduction.

As shown in the supplemental figure (Fig EV5 in the revised manuscript), our results are incompatible with those by Kuchay et al. regarding whether FBXL2 is a substrate of GGTase-III. In our analysis, FBXL2 can still localize to the plasma membrane in PTAR1 KO cells (Fig EV5A), while Kuchay et al. show cytosolic localization of FBXL2. This could be due to the difference in experimental conditions: we used PTAR1 KO cells, while Kuchay et al. used PTAR1 knock-down cells. Since we rigorously validated the absence of PTAR1 in the KO cells by direct genomic sequencing (Fig EV3B), immunoblotting (Fig EV3C), and the lack of GGTase-III activity (Fig 6C and Fig EV3E), our data provide clear evidence that GGTase-III is dispensable for FBXL2 localization to the membrane.

Most importantly, we could not detect FBXL2 geranylgeranylation by GGTase-III (Fig EV5C). We carefully confirmed this result using highly purified recombinant FBXL2-Skp1 complex (Fig EV5B). Thus, our results contradict the key finding of Kuchay et al. Although we cannot resolve this discrepancy, there are significant differences in experimental procedures between our study and Kuchay et al.

1) We examined prenyltransferase activity by measuring acid-precipitable radioactivity trapped on glass microfiber filters. This is a reliable measurement method that has been commonly employed by researchers in this field. In contrast, the Methods section of Kuchay et al. shows that they measured radioactivity blotted on chromatography papers.

2) The incubation time of their assays is unusually long (1.5 h). Despite this long incubation, GGTase-III-specific incorporation of geranylgeranyl is only about 2.5 times above the background (Kuchay et al. Fig. 2b). In addition, the vertical axis is expressed in x 10^{-6} µM/min, which appears to suggest that the enzyme reaction is negligibly slow. Furthermore, there are inconsistencies and ambiguities in their figures. For example, GGTase-III activity against 10 µM FBXL2 is ~8.5 x 10^{-6} in Fig 2a but ~3.5 x 10^{-6} in Fig 2b. It is also unclear whether they subtracted the high background counts when analyzing the data in Fig 2a.

3) Kuchay et al. claim that FBXL2 is preferred by GGTase-III over GGTase-I. However, the FBXL2 C-terminus is CVIL, which is the same as that of Rap1B. This indicates that FBXL2 is a good substrate for GGTase-I (CVIL peptide is used as a model substrate for GGTase-I in a crystallographic analysis; Taylor et al., EMBO J, 2003). Indeed, we observed highly efficient geranylgeranylation of FBXL2 by GGTase-I, which was saturated within 10 min (Fig EV5C). In contrast, Kuchay et al. show only a modest increase

in FBXL2 geranylgeranylation by GGTase-I (~600 cpm (with GGTase-I) vs ~350 cpm (no enzyme); Kuchay et al. Supplemental Fig 2b). In our measurements, FBXL2 geranylgeranylation after 10 min incubation was 10762 cpm (with GGTase-I) vs 209 cpm (no enzyme) (modified from Fig EV5C). These results again suggest that there are substantial experimental differences in our study and Kuchay et al.

In addition, they use commercially purchased recombinant GGTase-I (from Merck) to compare its activity with that of GGTase-III. This may lead to underestimation of GGTase-I activity against FBXL2 (In our analysis, GGTase-I has the highest catalytic activity among the four prenyltransferases; Fig EV1).

Collectively, these experimental differences may have led to the inconsistencies between our data and Kuchay et al. Another point is that, in the Kuchay paper, direct assessment of GGTase-III prenyltransferase activity is limited to Fig 2a and 2b, and other parts (including point mutational analysis) rely on binding assays (i.e. co-IP) or indirect detection using Click-iT chemistry. These results confirm the interaction of FBXL2 and GGTase-III, but do not directly support their claim that FBXL2 is a GGTase-III substrate. Thus, at present, we consider that it is highly improbable that GGTase-III preferentially prenylates FBXL2 (in the CAAX cysteine).

As the reviewer points out, an alternative scenario is that GGTase-III geranylgeranylates the cysteines adjacent to the CAAX motif of FBXL2. While this model seems plausible, there are some differences compared with the case of Ykt6.

1) Ykt6 remains fully soluble after the CAAX farnesylation by intramolecular sequestration of the farnesyl group, enabling easy access of GGTase-III to the substrate. In contrast, FBXL2 translocates to the membrane compartments after the CAAX geranylgeranylation (Fig EV5A). Therefore, GGTase-III has to extract FBXL2 from the membrane to catalyze additional geranylgeranylation. Also, physiological significance of the additional prenylation is unclear since FBXL2 can become fully membrane-bound in the absence of GGTase-III (Fig EV5A).

2) GGTase-III can geranylgeranylate Ykt6 without the preceding farnesylation, albeit inefficiently (Fig 3D and 3E). This contrasts with the result that GGTase-III has no activity on FBXL2 in its unprenylated form (Fig EV5C).

We have been trying to test this alternative model, but currently, we have no data that support it. Probably the most appropriate way to test this possibility would be to directly analyze the lipid modification status of cellular FBXL2 by mass spectrometry.

As described above, there remain many confusing and unresolved issues about FBXL2. Thus, we would like to discuss these issues in the last part of the Discussion not to interrupt our story on Ykt6. Although I mentioned several experimental differences between our paper and Kuchay et al., we would like to focus on our own results in the revised manuscript to avoid speculative discussions about the discrepancies. As the reviewer suggested, we discussed the possibility of geranylgeranylation of the adjacent cysteines of FBXL2. Also, we acknowledged the Kuchay paper in the Introduction. We thank the reviewer again for suggestions and the improvement of our manuscript.

The revised version of the manuscript is as follows:

Introduction, page 4, lines 28-29.
"A recent structural study suggested that PTAR1 can act as a prenyltransferase α subunit (Kuchay et al., 2019); however, the molecular function of PTAR1 remains unclear."

Discussion, page 15, line 23, to page 16, line 2.
"Our unbiased approach led to the discovery of a single protein substrate for GGTase-III, but we cannot exclude the possibility that GGTase-III has other unknown protein substrates. A recent structural study by Kuchay et al. (2019) has shown that PTAR1 heterodimerizes with RabGGTβ and interacts with FBXL2, a regulatory subunit of a membrane-localized ubiquitin ligase complex. They further showed that GGTase-III can geranylgeranylate FBXL2, which terminates in the sequence CVIL. However, since CVIL is a typical substrate sequence for GGTase-I (Taylor et al., 2003), it remains uncertain whether FBXL2 is a physiological substrate for GGTase-III. In our analysis, FBXL2 was still able to localize to the plasma membrane in PTAR1 KO HeLa cells (Fig EV5A), indicating

that GGTase-III is dispensable for the membrane localization of FBXL2. Furthermore, we found that GGTase-III could not geranylgeranylate FBXL2 under conditions where GGTase-I robustly geranylgeranylated it (Fig EV5B and C). These results contradict the findings of the previous study regarding FBXL2 as a GGTase-III substrate. However, FBXL2 has additional cysteines adjacent to the CAAX motif (CRCCVIL), raising the possibility that these cysteines could be geranylgeranylated by GGTase-III once the CAAX cysteine is geranylgeranylated by GGTase-I. Further investigation is needed to determine whether FBXL2 is a physiological substrate for GGTase-III."

Referee #2:

It is rare that I get a paper that is as beautifully done as this one. Congratulations to the authors! This paper describes an entirely new class of prenyltransferase (named GGTase-III) that adds a geranylgeranyl group to the Golgi SNARE, Ykt6. The preferred substrate is a farnesylated Ykt6 and they show that the product in vitro and in cells is doubly modified. The authors include the structure of the product bound to the enzyme and functional studies in cells, showing under-glycosylation of LAMP protein and an altered Golgi complex structure by EM of knock out cells. The only question this reviewer has is whether there are any additional substrates for this enzyme. The authors could look in vitro at any cytosolic proteins that acquire biotin-precursor in reactions with and without the GGTase-III subunit, monitored by western blot with streptavidin. This is not essential for the present story but would be an interesting result. Overall, well done! -Suzanne Pfeffer (signed review).

Response: I thank you for highly appreciating our work. As you suggest, we repeatedly performed in vitro biotin-geranylation assays using statin-treated HeLa cell cytosol and also using PTAR1 KO HeLa cell cytosol (in which substrates of GGTase-III should remain unprenylated), but we were unable to find additional protein substrates. Thus, at present, we think Ykt6 is the only substrate for GGTase-III at least in HeLa cells.

Referee #3:

The manuscript „A SNARE geranylgeranyltransferase essential for the organization of the Golgi apparatus" by Shirakawa et al. reports the characterization of a putative prenyl transferase and the discovery of its target, the SNARE protein Ykt6.

Prenylation is an important post-translational modification occurring on the C-termini of specific proteins. So far, the enzymes Farnesyltransferase (FTase), Geranylgeranyltransferase I (GGTase I), and GGTase II have been identified and characterized biochemically, structurally, and physiologically. In this manuscript, the authors report on the identification of a previously unrecognized GGTase that they term GGTase III. They were able to identify its target, the SNARE protein Ykt6. In contrast to other prenyltransferases, GGTase III can only prenylate Ykt6 after the processing of the target by 3 preceding post-translationally modifying enzymes: FTase, RCE, and ICMT. Hence, the C-terminus of Ykt6 gets farnesylated, proteolytically processed, and methylated before addition of the geranylgeranyl moiety by GGTase III.

The authors present a convincing study regarding the cellular function of GGTase III, its target identification, the structural features of the enzyme-target-complex, and the biochemical mechanism of geranylgeranyl transfer. I have enjoyed reading this work very much and I do not have any reservation concerning its publication. It will be interesting to see whether GGTase III may also have alternative targets. Previously it was thought that only small GTPases of the Rab family can be double prenylated (by GGTase II). However, this paper unexpectedly shows that also other and totally unrelated proteins (i.e. Ykt6) can be subject to double prenylation. This work may therefore form the basis for future studies on protein double prenylation. I would like to recommend publication of this paper enthusiastically.

Response: I thank the reviewer for appreciating our work. We are continuing to search for alternative targets of GGTase-III, but no additional substrates have been found so far. Therefore, we currently consider that Ykt6 is the only substrate for GGTase-III.

Minor comments:

- Regarding the title of the manuscript: The title implies generality of SNARE prenylation. I would suggest being more specific since Ykt6 is the only discovered target SNARE for GGTase III.

Response: I understand that it is better to make the title of the manuscript more specific. However, in the title of this paper, I would like to emphasize the new concept of "SNARE double prenylation", comparing with the previously established concept of "Rab double prenylation", so I would like to keep the current title for our manuscript. I thank the reviewer for this suggestion.

- Please refrain from claims of novelty (e.g. "novel type of protein prenylation"). Prenylation in general and geranylgeranylation are known for many years. Also, the structure of GGTase III is homologous to other prenyl transferases. Thus, the finding is scientifically interesting, but not truly novel.

Response: According to the reviewer, we changed the phrase "novel type of protein prenylation" in the Abstract and Discussion as follows:

Abstract, page 2, lines 14-15.
"Our findings reveal a fourth type of protein prenyltransferase that generates geranylgeranyl-farnesyl Ykt6."

Discussion, page 13, lines 2-3.
"--- Ykt6 undergoes sequential prenylation by FTase and the newly characterized enzyme GGTase-III, ---"

Discussion, page 16, line 3.
"We have discovered a fourth type of protein prenyltransferase, GGTase-III."

Accepted 31st January 2020

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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Corresponding Author Name: Ryutaro Shirakawa, Shuya Fukai

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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 χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Experiments were performed with three biological replicates unless otherwise noted. The sample size was chosen according to the standard of the field.
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?	No samples were excluded.
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.
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.	Most of the results were confirmed by two investigators independently.
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?	Most of the data were expressed as mean ± standard error of the mean (SEM) and not statistically analyzed.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA

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

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).	Clone numbers of commercial antibodies used in this study were described in the Materials 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.	Sources of cell lines were described in the Materials and Methods. The cells were routinely 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.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The protein data bank (PDB) accession codes for the crystallographic data odtained in this study were described in the Data Availability section of the manuscript.
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).	This study does not contain large datasets.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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