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WRN helicase and mismatch repair complexes independently and synergistically disrupt cruciform DNA structures

Petr Cejka, Valentina Mengoli, Ilaria Ceppi, Aurore Sanchez, Elda Cannavo, Swagata Halder, Sarah Scaglione, Pierre-Henri Gaillard, Peter McHugh, Nathalie Riesen, and Piergiorgio Pettazzoni
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Thank you for submitting your study on WRN and MMR complexes in the resolution of repeat-associated cruciform structures to The EMBO Journal. It has now been assessed by three expert referees, whose comments are copied below for your information. I am happy to say that all reviewers appreciate the interest and timeliness of the findings and the general quality of the work, and that we would therefore be interested in pursuing the work further for publication. As you will see, the reports do still bring up a number of specific queries and constructive criticisms, which I would invite you to answer/address in a revised version of the manuscript.

Referee #1:

In this manuscript, Valentina Mengoli and colleagues investigated on the mechanism behind the observed MMR-WRN synthetic lethality reported in CRC. Using elegant biochemistry experiments, they demonstrated that WRN is very active in melting cruciform structures through its helicase domain assisted by the RQC region and that WRN structurally assist MMR proteins in the unfolding of cruciform-containing DNA. Furthermore, the authors provide evidence that WRN-mediated unfolding of cruciform structures prevents targeting by SLX1-SLX4CCD nuclease activity and, finally, provide clues on why another RecQ helicase with high activity on cruciforms, BLM, is not synthetic lethal with the MSI phenotype in CRC.

The manuscript is clearly timely and significant since the observed WRN-MSI synthetic lethal interaction is highly significant for basic and translational reasons. The actual molecular mechanisms underlying th eobserved synthetic lethality is just emerging and is almost obscure why it is not observed under acute conditions and outside CRC MSI+ backgrounds.
So, findings from Valentina Mengoli and colleagues' work are very interesting for the field and well-suited for publication in EMBO Journal.

Indeed, although the topic has been already investigated by another group, the reason why loss of WRN should end-up in DNA breakage in that specific background, given that WRN is a so relevant and general replication caretaker, was unknown.

Biochemistry is neat and experiments well disegned and executed. Results are clear cut and support conclusions so I am happy to say that, basing on my expertise, I do not have any major concerns and I do not see any major revision to ask for.

I have only a minor point concerning BLM vs. WRN.
Both are very active towards cruciforms but BLM is no synthetic lethal. I agree with authors' conclusion that the in vitro activity does not fully recapitulate what happens in the cells, as suggested by the 3A-ATR mutant of WRN basically being as active as wild-type WRN. However, I would stress more this concept in the text to do not dampen the values of the findings.

Another minor comment is for figure 7. I think it might be useful to check what happens with BLM. It is a control but any difference might also contribute to outline why WRN but not BLM is so important in MSI+ CRC.

Referee #2:

Mengoli et al. have performed a study in which they biochemically characterize WRN and the core mismatch repair complexes' (MutSa, MutSB, and MutLa) abilities to resolve cruciform structures in vitro. They develop a system in which DNA cruciforms can be formed in plasmid DNA and then resolved by purified recombinant proteins. They demonstrate that WRN's helicase domain is important for cruciform resolving. Furthermore, BLM is also able to unwind cruciforms in vitro (as previously shown), suggesting that indeed in vivo it is WRN's recruitment to cruciforms, in addition to its helicase function, that is essential for cruciform stability in MSI cancer cells. They then seek to understand why complementation of MMR factors into MSI cancer cells somewhat reduces their dependency on WRN. They test the cruciform unwinding ability of all the major MMR complexes and demonstrate that MutSa, MutSB, and to a lesser extent, MutLa and MutLy can unwind cruciforms in vitro. This is a quite novel observation. This unwinding activity is independent of ATP and RPA, making its mechanism different from that of WRN. Lastly, they show that the unwinding functions of WRN and MMR complexes are synergistic, at least in vitro. Overall, the work is solid and timely. If they were to provide additional insight into how MMR complexes unwind DNA cruciforms, this would make their findings even more interesting. However, I don't consider this extension to the work essential for publication.

Suggestions:

1. The authors demonstrate that the MutS complexes both can unwind cruciforms well without the presence of ATP. Are there any other examples of helicases/translocases that don't consume ATP in their function?
2. Can the authors perform an analysis of the various sections of the MMR proteins necessary for cruciform resolving? This could be through purifying truncated proteins (for example, proteins lacking the C-terminus with its ATPase domain). This is not a requirement but might add mechanistic insight.
3. In Figure 7, the authors nicely demonstrate that WRN, even helicase-dead WRN, can stimulate MutSB or MutSa unwinding of cruciform structures. Can they probe this functional interaction further? Do these complexes bind the Holliday junction structure at the same time as WRN, or is binding mutually exclusive? Can they perform electrophoretic mobility shift assays with WRN and MutSB or MutSa? If WRN plays a structural role in helicase resolution, as they suggest, it would be interesting and important to understand how.
4. Is WRN's ability to stimulate MutSB or MutSa cruciform unwinding activity unique to WRN? Does BLM or another RecQ helicase also stimulate MutS complexes?
5. The authors found that BLM can also unwind cruciform DNA in vitro. In vivo, however, BLM does not appear to be essential for MSI cancer cells. It is possible, though, that BLM is acting as a (not very efficient) backup for WRN in vivo. Since loss of WRN produces a very dramatic phenotype on its own, it becomes almost impossible to test the combinatorial loss of both BLM and WRN. Can BLM stimulate (but is not essential for) the ability of WRN to unwind (TA)n cruciform DNA in vitro?

Referee #3:

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The experiments are thorough, well-controlled and thoughtfully evaluated. Alternative interpretations are considered where appropriate and addressed throughout. While one would like to know more about the unfolded product(s) created by WRN and MMR proteins, this is an excellent initial study.

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1. While most of the experiments are well-controlled using proteins with defined defects, there is one exception that requires addressing. Fig 7 shows TA repeat-cruciform unfolding is enhanced in the combination of WRN + MutLalpha. ATPase dead WRN still gave this enhancement, but the converse experiment with endo dead MutLalpha was not included. This experiment should be added to address the possibility that latent endo activity of MutLalpha on the cruciform is stimulated by WRN, thereby relieving supercoiling through nicking, and thereby indirectly destabilizing the cruciform.

2. A minor issue is that the source of the random IR DNA sequence was not clearly described. Is this a naturally occurring sequence (if so, which?) or is it a synthetic design?

Manuscript EMBOJ-2022-111998

Answers to referees

We would like to thank the reviewers for their interest in our manuscript and for providing helpful comments and suggestions. Below is our point-by-point response.

Please note that we have slightly changed the manuscript title, from "WRN and mismatch repair complexes directly and synergistically unfold cruciform structures", into: "WRN and mismatch repair complexes independently and synergistically disrupt cruciform structures" For more details, See Q1 of reviewer 2.

Referee #1:

In this manuscript, Valentina Mengoli and colleagues investigated on the mechanism behind the observed MMR-WRN synthetic lethality reported in CRC. Using elegant biochemistry experiments, they demonstrated that WRN is very active in melting cruciform structures through its helicase domain assisted by the RQC region and that WRN structurally assist MMR proteins in the unfolding of cruciform-containing DNA. Furthermore, the authors provide evidence that WRN-mediated unfolding of cruciform structures prevents targeting by SLX1-SLX4CCD nuclease activity and, finally, provide clues on why another RecQ helicase with high activity on cruciforms, BLM, is not synthetic lethal with the MSI phenotype in CRC.

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Answer: We now clarify the point better in the text.

Another minor comment is for figure 7. I think it might be useful to check what happens with BLM. It is a control but any difference might also contribute to outline why WRN but not BLM is so important in MSI+ CRC.

Answer: We tested for a functional interaction of BLM with MutSα, MutSβ, and MutLα. We observed that BLM behaved very similarly to WRN (see below, Fig R1 A-B). While this result was somewhat unexpected, we know from the literature that BLM physically and functionally interacts with mismatch repair proteins (BLM, unlike WRN has a MIP motif mediating interaction with MLH1, PMID 34330701). In our pulldown experiments, the interaction of MutLα with BLM and WRN was very similar (Fig R1 C). We therefore assume that as the WRN 3A mutant, BLM fails to be recruited to the proper structures on chromosomal DNA, despite in principle being able to replace WRN based on its biochemical activity. As reviewer #2 points out, testing for a residual (minimal) function of BLM in WRN-deficient cells is nearly impossible because WRN knockout causes already a very strong phenotype.

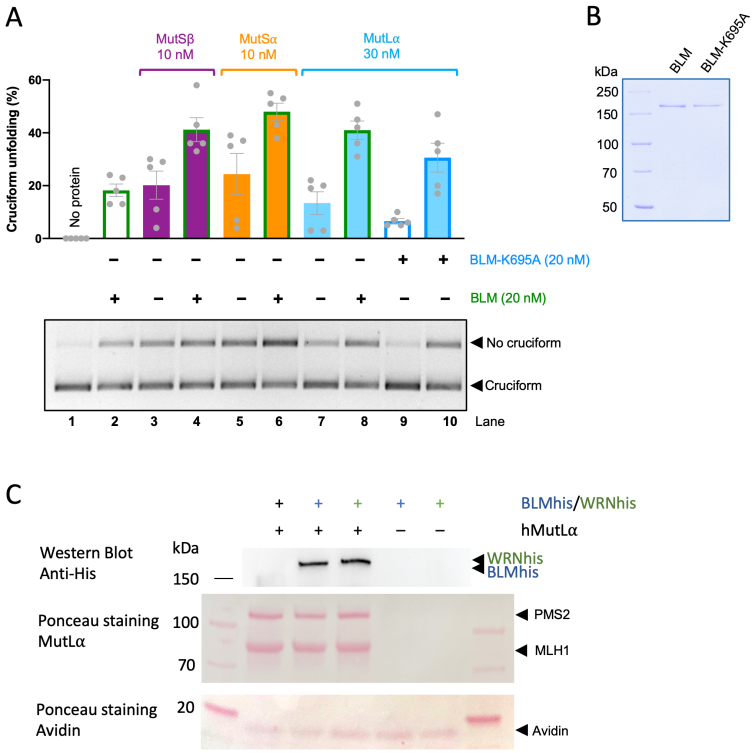


Fig R1 (A) Unfolding of TA cruciform by MMR proteins and wild-type BLM or helicase-dead BLM-K695A. Bottom, representative experiments; top, quantitation, averages shown; n = 5; error bars, SEM. (B) Representative polyacrylamide gel showing recombinant BLM and BLM-K695A. The gel was stained with Coomassie Brilliant blue. (C) Pulldown experiments where FLAG-tagged MLH1-PMS2 was immobilized on anti-FLAG affinity resin and incubated with equal amounts (0.5 μg) recombinant his-tagged WRN or BLM proteins. As a control, WRN and BLM were incubated with the resin without MLH1-PMS2. The experiment shows that MLH1-PMS2 interacts with both WRN and BLM with similar affinities.

Referee #2:

Mengoli et al. have performed a study in which they biochemically characterize WRN and the core mismatch repair complexes' (MutSa, MutSB, and MutLa) abilities to resolve cruciform structures in vitro. They develop a system in which DNA cruciforms can be formed in plasmid DNA and then resolved by purified recombinant proteins. They demonstrate that WRN's helicase domain is important for cruciform resolving. Furthermore, BLM is also able to unwind cruciforms in vitro (as previously shown), suggesting that indeed in vivo it is WRN's recruitment to cruciforms, in addition to its helicase function, that is essential for cruciform stability in MSI cancer cells. They then seek to understand why complementation of MMR factors into MSI cancer cells somewhat reduces their dependency on WRN. They test the cruciform unwinding ability of all the major MMR complexes and demonstrate that MutSa, MutSB, and to a lesser extent, MutLa and MutLy can unwind cruciforms in vitro. This is a quite novel observation. This unwinding activity is independent of ATP and RPA, making its mechanism different from that of WRN. Lastly, they show that the unwinding functions of WRN and MMR complexes are synergistic, at least in vitro. Overall, the work is solid and timely. If they were to provide additional insight into how MMR complexes unwind DNA cruciforms, this would make their findings even more interesting. However, I don't consider this extension to the work essential for publication.

Suggestions:

1. The authors demonstrate that the MutS complexes both can unwind cruciforms well without the presence of ATP. Are there any other examples of helicases/translocases that don't consume ATP in their function?

Answer: Canonical motor-driven helicases/translocases absolutely require ATP hydrolysis for DNA strand separation. There are however multiple examples of proteins that can trap ssDNA resulting from transient spontaneous DNA melting, such as RPA (e.g. PMID: 27016742). Other proteins, such the MRN complex can melt dsDNA, which is at least in part ATP hydrolysis independent (PMID 10346816). Likewise, also RAG1-RAG2 complex acts in an ATP-independent manner, and cuts dsDNA only after the two DNA strands are separated and

twisted by 180 degrees (PMID 32015552). In these cases, the ATP-independent enzymes exploit spontaneous and dynamic DNA structures.

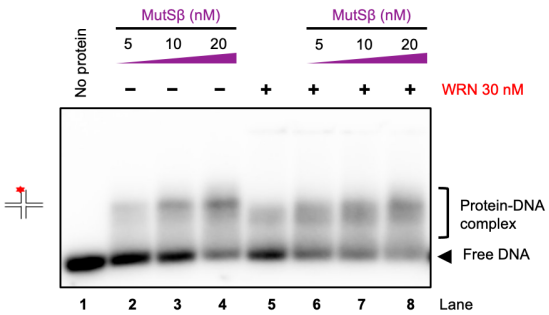
We now propose the following explanation, and thank the reviewer for making us thinking about this point: Holliday junctions (including cruciform DNA) are known to exist in several conformations, included folded and unfolded forms, that are highly dynamic (PMID 15824311). In search for a mismatch, the MutSα and MutSβ complexes adopt a "praying hands" structure, which forms a thermal diffusion-driven sliding clamp that scans dsDNA, independently of ATP (PMID 29792877). We believe that by encircling dsDNA, the complex exploits the dynamic structure of the cruciform to stabilize DNA in its double-stranded form. Therefore, the MMR complexes may not "melt" the structure *per se* as we initially proposed, but rather stabilize the dsDNA form resulting from spontaneous transitions. We have changed the wording in the text (including manuscript title) and discuss this model.

2. Can the authors perform an analysis of the various sections of the MMR proteins necessary for cruciform resolving? This could be through purifying truncated proteins (for example, proteins lacking the C-terminus with its ATPase domain). This is not a requirement but might add mechanistic insight.

Answer: The reviewer raises a valid point. We opted for several previously-characterized point mutants, which do not affect the stability of the heterodimers. We prepared MutLα mutant complexes (MLH1 E34A, PMS2 E41A), as well as MutSβ (MSH3 K902A) deficient in ATP binding. In agreement with our data that ATP was not required for the melting of the cruciform DNA by the MMR complexes (Fig. 6F), the mutations in the ATPase site did not affect the activity of the MMR complexes as well (Fig. 7A-F). Furthermore, we prepared the MutSβ complex with a mutation in MSH3 (K255A) (PMID 10938287), which specifically affects insertion-deletion loop recognition, but does not affect the binding of DNA with the cruciform structure (Fig. 7A-D). Also this mutant did not affect the ability of MutSβ to apparently melt cruciform DNA. We can therefore conclude that the MutS and MutL complexes function in an entirely MMR-independent manner. We plan to follow up on these experiments to further define the regions required for the apparent melting activity, and employ single-molecule approaches to tackle the mechanism in a future study.

3. In Figure 7, the authors nicely demonstrate that WRN, even helicase-dead WRN, can stimulate MutSB or MutSa unwinding of cruciform structures. Can they probe this functional interaction further? Do these complexes bind the Holliday junction structure at the same time as WRN, or is binding mutually exclusive? Can they perform electrophoretic mobility shift assays with WRN and MutSB or MutSa? If WRN plays a structural role in helicase resolution, as they suggest, it would be interesting and important to understand how.

Answer: We performed the electrophoretic mobility shift assays (see below Fig R2), and we failed to find evidence that WRN and MutSβ would bind DNA together to form a stable ternary complex. The EMSA however cannot detect transient interactions. Therefore, we cannot conclude whether there is a transient ternary complex or whether the proteins help to recruit each other but do not remain together once bound. We will need to employ novel mythologies to comprehensively address this question.



(Fig R2) Electrophoretic mobility shift assay with increasing concentrations of MutSβ, and increasing concentrations of MutSβ together with WRN, using a radioactively labelled HJ structure as a substrate. 0.8% unstained native agarose gel was used to separate the protein and DNA species.

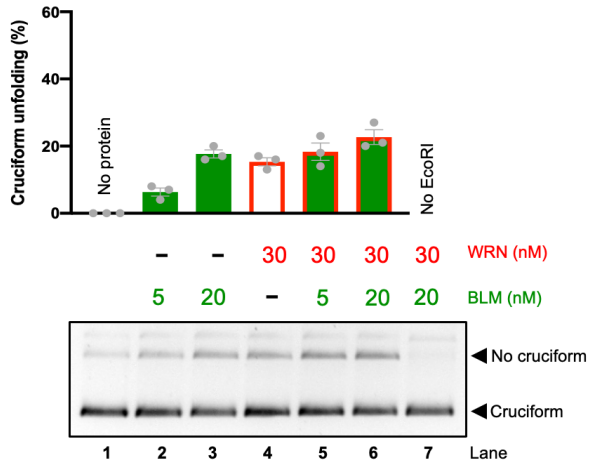
4. Is WRN's ability to stimulate MutSB or MutSa cruciform unwinding activity unique to WRN? Does BLM or another RecQ helicase also stimulate MutS complexes?

Answer: Please see the response to reviewer #1 above.

5. The authors found that BLM can also unwind cruciform DNA in vitro. In vivo, however, BLM does not appear to

be essential for MSI cancer cells. It is possible, though, that BLM is acting as a (not very efficient) backup for WRN in vivo. Since loss of WRN produces a very dramatic phenotype on its own, it becomes almost impossible to test the combinatorial loss of both BLM and WRN. Can BLM stimulate (but is not essential for) the ability of WRN to unwind (TA)n cruciform DNA in vitro?

Answer: We tested the combination of WRN and BLM in cruciform unfolding, using the TA-based cruciform as a substrate. There was no synergy; in fact, the combination of BLM and WRN was less than additive (compare lanes 2, 3 and 5 in Fig R3 below). Therefore, we think the helicases are unlikely to function together.



(Fig R3) Unfolding of TA cruciform by BLM, WRN, or BLM and WRN together. Bottom, representative experiment; top, quantitation, averages shown; n = 3; error bars, SEM.

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Concerns
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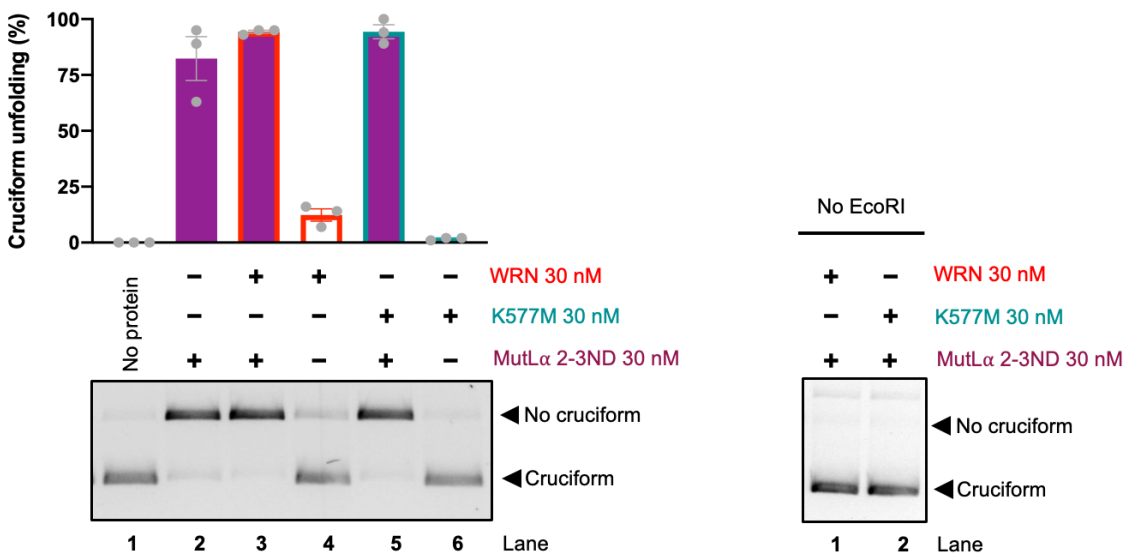
Answer: The reviewer raises an important point, as a latent nuclease activity (or a contamination) might indirectly destabilize the cruciform. We have comprehensively tested all proteins used in our assays under the same reaction conditions but without EcoRI (Fig. EV1B, WRN and variants; Fig. EV5B, all MMR MutS and MutL complexes). As can be seen from the panels, no nicked or linear DNA was observed, so we can confidently exclude contaminating nuclease activities. Of note, the assay contains Mg^{2+} as a metal co-factor, which does not support the nuclease activity of MutLα or MutLγ acting on their own (PMID 16873062, 32814904).

Nevertheless, we have also performed the experiment with nuclease-dead MutLα (3ND, mutations D699N, Q700K, E705K in the PMS2 subunit). The mutant variant also promotes cruciform unfolding (Fig R4).



(Fig R4) Left panel: Unfolding of TA cruciform by MutLα 2-3ND (PMS2 with mutations D699N, Q700K, E705K), *WRN, or WRN K577M. Bottom, representative experiment; top, quantitation, averages shown; n = 3; error bars, SEM. Right panel: Unfolding of TA cruciform by MutLα 2-3ND together with WRN, or together with WRN K577M, without addition of EcoRI, as a control of MutLα 2-3ND nuclease activity.*

2. A minor issue is that the source of the random IR DNA sequence was not clearly described. Is this a naturally occurring sequence (if so, which?) or is it a synthetic design?

We believe the random IR sequence is of a synthetic origin, it was first used by D. Lilley and later by S. West to study HJs and HJ resolvases. The plasmid was re-prepared from scratch based on pUC19, but we now also include the original reference for the respective sequence in the manuscript.

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that in light of the positive re-review by one of the original referees (copied below), we have now accepted it for publication in The EMBO Journal.

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The revised manuscript answers all my concerns satisfactorilly.

EMBO Press Author Checklist

Corresponding Author Name: Cejka
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Manuscript Number: EMBOJ-2022-111998

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 $\chi 2$ 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	No restrictions on easily replicable material (plasmids), not easily replicable material (purified proteins) depending on availability. The information is abailable in Material and Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Not Applicable	

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Appendix Table EV1, Appendix Table EV2

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure Legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	Blinding is not applicable in biochemical assays.
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	In our biochemical assays, generally, we did not exclude any data, except in cases when there were suspected problems (postive/negative controls not working as expected), or in cases of other technical issues (broken wells, gels, etc.). We do not think that a statement in the text would be relevant.
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	We believe that our statistical analyses are adequate (in our case, this refers to error bars). Error bars are defined in Figure Legends.

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Number of experiments is always defined in Figure Legends, additionally, we present all individual data as datapoints in our bar graphs.
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure Legends.

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	Our study does not contain large datasets, so we did not use data deposition. Our manuscript includes "Data availability: This study includes no data deposited in external repositories." All primary data are available upon request.
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	