Mime-seq 2.0: a method to sequence microRNAs from specific mouse cell types

Ariane Mandlbauer, Qiong Sun, Niko Popitsch, Tanja Schwickert, Miroslava Spanova, Jingkui Wang, Stefan Ameres, Meinrad Busslinger, and Luisa Cochella

Corresponding authors: Luisa Cochella (mcochel1@jhmi.edu) , Stefan Ameres (stefan.ameres@univie.ac.at), Meinrad Busslinger (Meinrad.Busslinger@imp.ac.at)

Review Timeline:

Editor: Yehu Moran

Transaction Report:

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Dear Dr. Cochella,

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three expert referees whose comments are provided below.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing in detail the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

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

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Yehu Moran
Academic Editor
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (6th May 2024). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

In this manuscript, A. Mandlbauer and colleagues describe an improvement of the "mime-seq" technique. That technique allows cell-specific sequencing of miRNAs without the need for cell sorting (rather using an elegant method based on miRNA methylation, where the methylase is expressed in the cell type of interest; sequencing methods allow the selective detection of methylated miRNAs only, therefore enriching for miRNAs from that cell type). This is a much-needed method, addressing most of the issues the community is facing with single-cell sequencing in Small RNA-Seq - and therefore the topic is of high technical interest. The original technique (published by the same labs in 2018) was developed for D. melanogaster and C. elegans, but it did not work in mammalian cells. The current manuscript describes an adaptation of the method for mammalian cells (basically, a mammalian methylase has to be tethered to the Argonaute partner of miRNAs for the methylation reaction to work efficiently in mammalian cells). The manuscript is well written, it is very clear and the data is convincing. I could only spot a few minor defects, which should be easy to fix before the manuscript is acceptable for publication.

Minor points:

1. Figure 1D: It is not fully clear what the "+" and "-" signs indicate (with or without oxidation treatment?). Please clarify.

2. Figure 2C: axis legends are very confusing. I cannot make sense of the "-" signs, they don't appear to be "minus" signs. What do the authors mean when they wite "normalized reads - unox" and "UT - log10(normalized reads)"? If this sign is just a meaningless delimiter (meaning something like "normalized reads, unox") then the axis titles would make much more sense; if it is the case, then please use another symbol than a minus sign.

3. Legend for Figures 2 B-E: "MicroRNAs with spike-in-normalized reads <0.01 were removed from the analysis" (same thing on main text, line #160). This is quite obscure. What does this number represent exactly? The amount of introduced spike-in oligos should be given (I could not find it), and if this "0.01" value is a cutoff value on the ratio between read numbers for miRNAs and one of the spike-ins (or the sum of all of them?), then please say it explicitly.

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Mandlbauer et al.

Mime-seq 2.0: a method to sequence microRNAs from specific mouse cell types

In this manuscript, the authors have further developed a tissue-specific miRNA sequencing method called Mime-seq. This is an elegant method that uses the plant methyltransferase Hen1 in C. elegans and flies to methylate miRNAs. This, however, did not work for mammalian cells. Therefore, the authors used HENMT1 that is naturally expressed in mouse germ cells and functions in the piRNA pathway. They generated a C-terminally truncated version and fused it to an Argonaute binding peptide termed T6B. Using this approach, the methyltransferase is targeted to Ago proteins and methylates the bound miRNA. An adapted sequencing protocol that oxidizes non-methylated miRNAs can be used to specifically detect the modified miRNAs. This approach worked well in tissue culture cells and thus the authors produced an inducible mouse allowing for the expression of the

T6B-HENMT1 in specific tissues and particularly in rare tissues and cells. The authors investigated a number of rare cells from the hematopoietic lineage and were able to enrich and identify cell-type specific miRNAs with high specificity. Even highly diluted cell numbers led to an accurate retrieval of the cell-specific miRNAs.

This is a very clear and straightforward study. It is well presented and provides an elegant tool for the field. Particularly the mouse model will be highly appreciated and allows for accurate miRNA profiling without prior cell sorting. I did not find any technical issues or problems with this study. I have only one point/comment that should be considered.

The fact that overexpression of the T6B-HENMT1 in the B cell lineage does not cause any effects on B cell development is somewhat surprising. The T6B peptide should lead to a global miRNA inhibition and this has been associated with hematopoietic cell development. However, an explanation could be that the T6B-fused enzyme is only inefficiently bound to Ago proteins but nevertheless methylates miRNAs even in an Ago-unbound state. To control for that, a non-fused HENMT1 should be overexpressed. A mutated and Ago-binding-deficient T6B peptide could also be used. This should at least be discussed in detail.

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In the manuscript, the authors describe mime-seq2, a variant of their previously described approach based on artificially methylating miRNAs within cells by expressing a particular transgene and then reading out the methylation status of miRNAs post-extraction. Overall, this is a very clever and efficient approach. Mime-seq was developed for nematode and fly cells, and here the authors mention that the original approach does not work in mammalian cells, likely due to the more limited availability of "naked" Dicer products. Instead, they utilize an alternative methylation protein that they fuse to an Ago-interacting peptide and show in cells and in a mouse model that this approach can indeed methylate miRNAs in culture and in blood cells and can be indeed used to identify cell-type-specific miRNAs expressed in a small subpopulation of cells in vivo, given that a suitable Cre driver is available for that population.

The paper is very well written, and the results are carefully and informatively presented. The approach is clever, though the innovation is primarily technical rather than conceptual, as the concept was already introduced in mime-seq. My only yet major concern is that I strongly wonder if the method will be used by more than a handful of other labs. The authors correctly mention that the breeding scheme for introducing the methylating peptide is relatively simple. Still, any breeding of mouse models introduces considerable work and costs, and if breeding is needed, potential users can just breed their mice to a Cre-driven GFP model, and sort and sequencing the positive cells. This is bound to give more information as spatial examination of the GFP signal will also be available, other RNA types can sequenced, etc. As the authors note, their method is only semi-quantiative, and so mostly relevant in cases where a particular miRNA is really cell-type-specific, but in these cases it will be easy to study also by sequencing sorted cells. Also, most functionally relevant miRNA in mice are already probably known, and I doubt that mime-seq2 will be able to find many additional and highly specific miRNAs. The results presented in the manuscript also do not really show a use-case scenario where mime-seq2 is giving insights that were not previously accessible. As such, this manuscript is mostly a technical tour-de-force, and sets a high technical standard, but its impact on the miRNA community will likely be limited, and as such it might be more suitable for a more specialized journal than EMBO J.

Point by point response to the reviewers: our replies are in blue

We thank all three reviewers for their time, comments and advice on how to improve our work.

Referee #1:

In this manuscript, A. Mandlbauer and colleagues describe an improvement of the "mime-seq" technique. That technique allows cell-specific sequencing of miRNAs without the need for cell sorting (rather using an elegant method based on miRNA methylation, where the methylase is expressed in the cell type of interest; sequencing methods allow the selective detection of methylated miRNAs only, therefore enriching for miRNAs from that cell type). This is a much-needed method, addressing most of the issues the community is facing with single-cell sequencing in Small RNA-Seq - and therefore the topic is of high technical interest. The original technique (published by the same labs in 2018) was developed for D. melanogaster and C. elegans, but it did not work in mammalian cells. The current manuscript describes an adaptation of the method for mammalian cells (basically, a mammalian methylase has to be tethered to the Argonaute partner of miRNAs for the methylation reaction to work efficiently in mammalian cells). The manuscript is well written, it is very clear and the data is convincing. I could only spot a few minor defects, which should be easy to fix before the manuscript is acceptable for publication.

Minor points:

1. Figure 1D: It is not fully clear what the "+" and "-" signs indicate (with or without oxidation treatment?). Please clarify.

We apologize for the lack of a clear annotation in the figure. The signs referred to whether the sample had been oxidized and beta-eliminated ("+") or not ("-"). This information has now been added to the revised figure 1.

2. Figure 2C: axis legends are very confusing. I cannot make sense of the "-" signs, they don't appear to be "minus" signs. What do the authors mean when they wite "normalized reads - unox" and "UT - log10(normalized reads)"? If this sign is just a meaningless delimiter (meaning something like "normalized reads, unox") then the axis titles would make much more sense; if it is the case, then please use another symbol than a minus sign.

We see the problem with our previous labeling and the dash. The axes have been relabeled to indicate the relevant comparison in that figure: non-transduced vs. HENMT1-T6B-expressing cells, all under unoxidized conditions (stated in the legend).

3. Legend for Figures 2 B-E: "MicroRNAs with spike-in-normalized reads <0.01 were removed from the analysis" (same thing on main text, line #160). This is quite obscure. What does this number represent exactly? The amount of introduced spike-in oligos should be given (I could not find it), and if this "0.01" value is a cutoff value on the ratio between read numbers for miRNAs and one of the spike-ins (or the sum of all of them?), then please say it explicitly.

We appreciate the reviewer highlighting this ambiguity. To exclude miRNAs with low confidence of expression (and thus considered biologically insignificant) from our analysis, we implemented a threshold. Specifically, we set a cutoff at 0.01 normalized counts based on spike-in normalized reads in RKO cells and 0.5 in all mouse samples. This cutoff, informed by our spike-in normalization (detailed in the note on spike-in concentrations), equates to 2 attomoles (amol) of miRNA per microgram (µg) of total RNA. Consequently, this translates to fewer than 15 miRNA molecules in RKO cells and in our mouse samples, considering the total RNA content per cell is approximately 10 picograms (pg) for RKO and about 1-4 pg for our mouse samples. These figures are in stark contrast to the most abundant miRNAs, such as miR-21-5p, which exists in approximately 42,000 copies per RKO cell, demonstrating that our chosen threshold is conservative.

We have added this rationale to the methods section (lines 506-510) and have updated Table EV1 with the spike-in concentrations utilized and have specified the spike-in

oligonucleotides employed in the Methods section. The range of spike-in concentrations spans from the lowest at 10 amol/µg of total RNA (approximately 60 molecules per cell) to the highest at 5,000 amol/µg of total RNA (approximately 30,110 molecules per cell).

4. Figure 2E: the figure legend mentions a correlation coefficient, which does not appear on the plots.

We apologize for the oversight. The correlation coefficients have been reinstated in the figure.

5. Figures 3 B and C: "Statistical data (B,C) were analyzed by one-way ANOVA with Dunnett's multiple comparison test". The method employed here makes perfect sense, but one piece of information is missing: which condition has been used as a control in Dunnett's test? Intuitively I would have expected it to be the HenT6B/HenT6B condition (black bars), but this is not consistent with the fact that some of the asterisks shown span only the purple and blue bars. Please clarify.

We thank the reviewer for pointing out this inconsistency. By mistake, we compared all data with the B cell type of the heterozygous (*Cd79a*-Cre HenT6B/+) genotype. In the meantime, we have performed two additional flow-cytometric experiments with heterozygous (*Cd79a*-Cre HenT6B/+), homozygous (*Cd79a*-Cre HenT6B/HenT6B) and control (HenT6B/+ or HenT6B/HenT6B) mice. We now examined at least 10 mice of each genotype in 3 independent experiments, which has significantly improved the statistical analysis. We have analyzed the new flow-cytometric data by comparing the data relative to the control genotypes (HenT6B/+ or HenT6B/HenT6B) with one-way ANOVA with Dunnett's multiple comparisons test. Due to the analysis of more mice, each B cell type is now shown to be present at similar frequency in the bone marrow of the different genotypes.

6. Materials and Methods, section "RNA extraction and preparation": "sequences are provided in supplementary table X", with X being an apparent placeholder that needs to be corrected.

Indeed, the reference to "Table X" was intended to direct readers to Table EV1. This has now been corrected.

Referee #2:

Mandlbauer et al.

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In this manuscript, the authors have further developed a tissue-specific miRNA sequencing method called Mime-seq. This is an elegant method that uses the plant methyltransferase Hen1 in C. elegans and flies to methylate miRNAs. This, however, did not work for mammalian cells. Therefore, the authors used HENMT1 that is naturally expressed in mouse germ cells and functions in the piRNA pathway. They generated a C-terminally truncated version and fused it to an Argonaute binding peptide termed T6B. Using this approach, the methyltransferase is targeted to Ago proteins and methylates the bound miRNA. An adapted sequencing protocol that oxidizes non-methylated miRNAs can be used to specifically detect the modified miRNAs. This approach worked well in tissue culture cells and thus the authors produced an inducible mouse allowing for the expression of the T6B-HENMT1 in specific tissues and particularly in rare tissues and cells. The authors investigated a number of rare cells from the hematopoietic lineage and were able to enrich and identify cell-type specific miRNAs with high specificity. Even highly diluted cell numbers led to an accurate retrieval of the cell-specific miRNAs.

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The reviewer highlights an important point regarding a seeming discrepancy between a previously published study, in which overexpression of a T6B-YFP fusion protein caused an increase in pro-B cell numbers and a strong decrease in pre-B, immature and mature B cell numbers in the bone marrow of transgenic mice (La Rocca *et al. e*Life, 2021). In marked contrast, we observed similar numbers of each B cell type in the bone marrow of control (HenT6B/+ or HenT6B/HenT6B), heterozygous (*Cd79a*-Cre HenT6B/+) and homozygous (*Cd79a*-Cre HenT6B/ HenT6B) mice (revised Figure 3B,C). La Rocca et al. proposed that T6B as part of T6B-YFP fusion protein acts as a competitive inhibitor of TNRC6-dependent gene regulation mediated by Ago-bound miRNAs. As such, the efficacy, with which T6B can compete with endogenous Ago-TNRC6 interactions, may be contingent on the extent of T6B overexpression and/or its affinity for Ago. It is plausible that the lower expression of HenT6B from the *Rosa26* locus (our study) compared to the doxycycline-induced expression of T6B-YFP from the *Col1a1* locus (La Rocca *et al.*, 2021) might explain the discrepancy of the observed B cell phenotypes. We have now included this consideration in a new paragraph within the discussion section (lines 261-271).

In response to the reviewer's suggestion to test miRNA methylation activity of non-T6B-fused HENMT1 variants, we examined the modification status of endogenous miRNAs upon expression of full-length HENMT1 and the truncated variant (HENMT1$^{\Delta C}$) – neither of which were fused to T6B – across three different cell types. In neither condition did we observe miRNA methylation (as shown in Figures 1 and EV1), strongly suggesting that miRNA methylation requires T6B-dependent association of HENMT1 with Ago. These findings and their implications are now detailed in lines 113-117 and 248-250 in our manuscript.

New paragraph for discussion:

When testing mime-seq 2.0 in B cell types of the mouse hematopoietic system, we found no indication of inhibition of miRNA function by HENMT1$^{\Delta C}$-T6B. Overexpression of a T6B-YFP fusion

protein was, however, previously shown to inhibit miRNA function by competing for binding of endogenous TNRC6 to Argonaute, although this did not affect the miRNA repertoire (La Rocca *et al*., 2021). Within the hematopoietic system, T6B-YFP overexpression under the control of a doxycycline-inducible promoter in the *Col1a1* locus resulted in an increase of pro-B cells and a decrease of pre-B, immature and mature B cells in the bone marrow (La Rocca *et al*, 2021). In contrast, we observed similar frequencies of all B cell types in the bone marrow upon B cell-specific expression of HENMT1$^{\Delta C}$-T6B from the *Rosa26* locus. Given that T6B acts as a competitive inhibitor, this discrepancy might be explained by differences in expression level, or binding affinity. In our study, the expression of HENMT1$^{\Delta C}$-T6B seems to be sufficient to induce the necessary levels of miRNA methylation without affecting the generation or viability of the cells expressing the enzyme in contrast to the overexpression of T6B-YFP protein.

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We appreciate the reviewer's opinion but respectfully disagree with some of the assessments based on the findings of our study:

Regarding the point that potential users may prefer to cross their specific Cre line to an inducible GFP transgene (such as Rosa26(LSL-GFP/+)), we would like to point out that our HENMT-expressing transgene also expresses GFP in a Cre-dependent manner. Hence, in addition to any advantage of visualizing transgene expression and facilitating cell sorting for miRNA sequencing, the HenT6B transgene further allow for miRNA analysis by mime-seq. We realize that the fact that our transgene includes a GFP reporter was not sufficiently pointed out. Therefore, we now mention this more explicitly in the text (lines 157-159). It is true that scientists working in the miRNA field, who have no access to mouse genetics expertise, will neither use our Rosa26(LSL-HenT6B-P2A-GFP/+) or the Rosa26(LSL-GFP/+) mouse strains. But for scientists with access to a mouse facility, the HenT6B transgene described here provides a considerable advantage.

The main advantage of our approach is that sorting will not be needed for miRNA sequencing. This is of significant advantage for a number of reasons: First, the enzymatic and mechanical treatments necessary for dissociation can introduce unknown changes due to cell stress, especially for tissue-embedded cells which may be damaged by this procedure. Second, for rare cell types, acquiring the necessary number of cells for high-quality libraries may require lengthy or even multiple sorting sessions. Finally, sorting requires access to a flow cytometer capable of sorting. Mime-seq overcomes these challenges and may provide a preferred solution relative to isolating GFP-positive cells.

We agree with the reviewer that most miRNAs in the mouse genome are most likely comprehensively annotated. We therefore do not expect that mime-seq will necessarily lead to the discovery of new miRNAs. However, even in the worm, where all miRNAs are definitely known, elucidating the precise patterns of expression and the specificity within a given tissue has been essential to fully understand their function. In general, miRNA profiling in the mouse is still performed at a surprisingly low resolution (complex tissues or even whole organs). We therefore anticipate that having a tool to gain such resolution will be useful for dissecting the function of even well-known miRNAs in different cell types.

Finally, only time will tell how broadly this mouse model will be implemented in the future. However, given comments from the other reviewers and the fact that we already received inquiries to ship the Rosa26(LSL-HenT6B-2A-GFP/+) mouse strain, we anticipate that this will be a useful new tool for the miRNA field.

Dear Dr. Cochella,

I am pleased to inform you that after going over your revised manuscript and your point-by-point response to the referees, I find your manuscript suitable for acceptance in principle. However, there are still few relatively small things that were caught by our editorial assistance team and need your attention (please see the list below my signature). Please make these corrections and re-submit your manuscript so we can formally accept it.

Congratulations and best wishes,
Yehu Moran

Academic Editor
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (25th Jun 2024). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

The authors addressed the minor editorial issues.

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Yours sincerely,

Yehu Moran
Academic Editor
The EMBO Journal

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The data shown in figures should satisfy the following conditions:

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- → Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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Each figure caption should contain the following information, for each panel where they are relevant:

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- → 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.

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

Materials

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	Mice or frozen sperm will be made available (Methods), HENMT1dC-T6B plasmid will be made available through addgene (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	Yes	Information can be found in the reagents and tools tables as well as indicated in the Materials and Methods section.

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Sequences and information on nucleotide modifications are provided in Table EV1.

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Cell line information can be found in the Reagents and Tools table.
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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.	Yes	Reagents and Tools
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and methods

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?	Yes	Core facility services are acknowledged in the acknowledgment section of the manuscript.

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.	Yes	Materials and Methods

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.	Not Applicable	
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	
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.	Yes	Sequencing reads below a threshold as stated in the manuscript were excluded (figure legends, materials and methods).
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	Methods and 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	Figure legends
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.	Yes	Materials and methods- Austrian Veterinary Authorities
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?	Yes	Sequencing raw data is deopsited via Zenodo depository as stated in Data Availability section. Accession numbers are provided in the text.
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?	Yes	All relevant processing pipelines are available through github. Accession links are provided in the Data availability section.
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	