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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software is used for data collection
Data analysis	Guppy (v6.3.7); Dorado (v0.7.2); Minimap2 (v2.22); Samtools (v1.10); bedtools (v2.26.0); Nanopolish (v0.13.2); f5c (v1.4); sam2tsv (v0558422); cutadapt (v4.1); seqkit (v0.8.1); fastp (v0.21.0); Star (v020201); Tombo (v1.5.1); Nanom6A (v2.0); DENA (first_release); m6Anet (v2.1.0); xPore (v2.0); m6ABasecaller (v1.0a); flair (v1.6.1); trmap (v0.12.6); Seqlogo (https://bioconductor.org/packages/release/bioc/html/seqLogo.html); DRIMSeq (3.17); Integrative Genomics Viewer (v2.12.3); All custom code will be available at https://github.com/xieyy46/SingleMod-v1.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

Sequencing data generated in this study can be found in GEO with the accession number GSE246632. Publicly available DRS datasets used in this study include: WT and METTL3 KO HEK293T (ENA: PRJEB40872); HeLa and K562 (PRJEB40872); WT and Mettl3 KO mESC (GEO: GSE195618 and SRP166020); WT and VIRILIZER mutant

A. thaliana (ENA: PRJEB32782); O. sativa (GSA: CRR521994); D. rerio (ENA: PRJEB53494); P.trichocarpa (GEO: SRR12822922); T. gondii (GEO: PRJNA705300); C.elegans (ENA: PRJEB31791); Curlcake (GEO: GSE124309); Oligomer data was obtained from the original author; SL-Oligomers (ENA: PRJEB74106). Publicly available NGS datasets used in this study include: GLORI in HEK293T and HeLa (GEO: GSE210563); eTAM-seq in mESC (GEO: GSE201064).

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	N/A
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The training and evaluation of SingleMod (RNA002) were conducted using nanopore DRS data and the corresponding GLORI data from multiple samples (HEK293T, HeLa, mESC, Arabidopsis seedlings, rice root, Chlamydomonas). The training and evaluation of SingleMod (RNA004) were conducted using nanopore DRS data from HEK293T and rice root. eTAM-seq data from mESC and DRS data from three types of m6A-control RNA molecules (Curlcakes, Oligomer and SL-Oligomers) were also included for SingleMod (RNA002) evaluation. In addition, DRS data from 5 other species (and K562) were included in the comparative analysis of multi-species single-molecule m6A profiles. No sample size calculation was performed, and all data used in this study have been published.
Data exclusions	We filtered low quality (qscore < 7) DRS reads based on the common and pre-established criteria in this field.
Replication	For SingleMod (RNA002) development, triplicate DRS datasets from HEK293T and HeLa were merged; quadruplicate DRS datasets from Arabidopsis were merged; duplicate DRS datasets from rice were merged; Chlamydomonas do not have replicate samples. For SingleMod (RNA004) development, no replicates were used. For comparative analysis of single-molecule m6A profiles across multi-species, no replicates were used.
Randomization	Samples were not randomization but covariates were controlled by running controls in parallel whatever applicable.
Blinding	Blindling was not relevant to this study, as the performance of these ONT tools was evaluated by comparing against to each other and the validation datasets.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☐	☒ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	Wild type (WT) of mouse 129 embryonic stem cells (mESCs) and Mettl3 knockout (KO) mESCs were provided by Dr. Jiekai Chen (Guangzhou Institutes of Biomedicine and Health, CAS) which had been validated in published work (Liu et al., Nature 591, 322-326, 2021). WT of mouse 129 mESCs were derived from 3.5 d.p.c inner cell mass (ICM) from female 129 mice (Stock NO.: 217, Beijing Vital River Laboratory Animal Technology Co., Ltd.); HEK293T cells (ATCC, passages <25) were cultured in DMEM (Corning; Cat#: 10-013-CVRC) supplemented with 10% fetal bovine serum (BIOVISION; Cat#: BVS500) at 37°C with 5% CO2.
Authentication	None of cell lines used were authenticated by authors.
Mycoplasma contamination	The cells were regularly tested for mycoplasma and certified negative.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell line used.

Plants

Seed stocks	The seeds of wild type Arabidopsis accession Col-0 were sown on MS10 medium plates, stratified at 4°C for 2 days, germinated in a controlled environment at 22°C under 16 hr light/8 hr dark conditions and harvested 14 days after transfer to 22°C; Rice (Oryza sativa L. subsp. japonica cultivar Nipponbare) seedlings were germinated and cultivated in an artificial climate chamber, where they were exposed to a temperature regime of 34°C during the day and 25°C at night. They were grown under a photoperiod of 16 hours of light followed by 8 hours of darkness, all on 1/2 Murashige and Skoog medium plates. The roots of these two-week-old seedlings were then carefully collected.
Novel plant genotypes	No novel plant genotypes were produced in this study.
Authentication	None of plants used were authenticated by authors.