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Reporting Summary

Nature Research 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 Research policies, see Authors & Referees 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	MicroManager (1.4.22)
Data analysis	Custom Mathematica code (11.3) and Fiji ImageJ

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/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research 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 list of figures that have associated raw data
- A description of any restrictions on data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. All code used to analyze the data set will be available on GitHub.

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

Life sciences study design

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

Sample size	Fig 1c: Representative image from n=39 independent biological replicates (cells). Fig 1d: n=39 independent biological replicates (cells) for the Original Tag and n=35 independent biological replicates (cells) for the NoIRES Tag. Fig 2b: Representative track out of 296 tracked Cap+IRES translation sites Fig 2c top: Avg of 296 tracked Cap+IRES translation sites from 11 independent biological replicates (cells). Most intense group has 99 tracked mRNA, the medium intense group has 99 tracked mRNA, and the least intense group has 98 tracked mRNA. Fig 2c bottom: Avg of 259 tracked Cap+IRES translation sites from 11 cells. Most intense group has 87 tracked mRNA, the medium intense group has 86 tracked mRNA, and the least intense group has 86 tracked mRNA. Fig 2d Top: Avg of 793 tracked Cap Only translation sites from 11 independent biological replicates (cells). Most intense group has 199 tracked mRNA, the most intense medium group has 198 tracked mRNA, the least medium intense group has 198, and the least intense group has 198 tracked mRNA. Fig 2d Bottom: Avg of 213 tracked Cap Only translation sites from 11 cells. Most intense group has 53 tracked mRNA, the most intense medium group has 54 tracked mRNA, the least medium intense group has 53, and the least intense group has 53 tracked mRNA. Fig 3a: Representative cell from the 17 independent biological replicates (cells) Fig 3b: n=17 independent biological replicates (cells). Fig 3c: n=10 independent biological replicates (cells). Fig 4a top: Representative cell of Original Tap out of 39 independent biological replicates (cells) Fig 4a bottom: Representative cell of Switch Tag out of 37 independent biological replicates (cells) Fig 4b from left to right: n= 302 spots out of 39 independent biological replicates (cells), n=167 spots out of 37 independent biological replicates (cells), n=262 spots out of 37 independent biological replicates (cells), n=201 spots out of 39 independent biological replicates (cells). Fig 4c from left to right: 226 spots out of 39 independent biological replicates (cells), n=76 spots out of 39 independent biological replicates (cells), n=121 spots out of 37 independent biological replicates (cells), n=76 spots out of 37 independent biological replicates (cells). Fig 5a: 14 unique 4 state models were considering with between 7 and 12 free parameters. The best model had 8 free parameters. Fig 5b-e: Model was simulated for 4,000 trajectories with a burn-in period of 10,000 seconds. Fig 5f: NaAs-n=32 independent biological replicates (cells). DTT-n=28 independent biological replicates (cells) Supplementary Fig 1c: n=5 (top, left), n=5 (top, right), n=6 (bottom, left), n=5 (bottom, right) independent biological replicates (cells). Supplementary Fig 2a: n=39 independent biological replicates (cells). Supplementary Fig 2b: n=39 independent biological replicates (cells). Supplementary Fig 2c: Representative cell of Original Tag out of n=11 independent biological replicates (cells). Below the image is a representative trace of a non-translating mRNA out of n=3771 total tracked mRNA translating and non-translating out of n=11 independent biological replicates (cells). Supplementary Fig 2d: n=3771 total tracked mRNA translating and non-translating from 11 independent biological replicates (cells). Supplementary Fig 3a: Representative data set out of n=296 translation spots from n=11 independent biological replicates (cells). Supplementary Fig 3b: Each point represents the median distance from the 3'UTR for either Cap in green of IRES in blue within n=296 Cap +IRES translation sites. Supplementary Fig 4c: n=7 independent biological replicates (cells). Supplementary Fig 4d: n=7 independent biological replicates (cells). Supplementary Fig 5a: n= 39 independent biological replicates (cells) for the Original Tag. n= 37 independent biological replicates (cells) for the Switch Tag. Supplementary Fig 5b: n=39 independent biological replicates for the Original Tag. n=37 biological replicates for the Switch Tag. Supplementary Fig 5c: n=47 spots for SM intensity Calibration from n=13 independent biological replicates (cells) and n=20 spots Cap Only in Original Tag from n=10 independent biological replicates (cells).
Data exclusions	Movies of cells that expressed too many reporter mRNAs were excluded because each mRNA cannot be tracked in these scenarios. For Harringtonine experiments, cells that were not expressing IRES-mediated translation were eliminated.
Replication	We confirmed reproducibility by performing at least 3 independent experimental replicates for each assay.
Randomization	This is not relevant to our study as we automated the analysis of microscopy data in an unbiased manner. We used consistent thresholding for mRNA and translation detection for each experiment.
Blinding	We used consistent thresholds to detect mRNAs and translation throughout all experimental conditions. Following detection, analysis was fully automated such that there was no bias from the experimenter involved.

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
☐	☐ Animals and other organisms
☐	☐ Human research participants
☐	☐ Clinical data

Methods

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

Antibodies

Antibodies used: Anti-DYKDDDDK antibody: Manufacturer: Wako, Catalog number: 012-22384, Clone No. 1E6, Lot number: SAN4130. 100 micrograms/mL of fluorescently labeled antibody fragment was used for each assay.

Validation: We generated FLAG Fab fragments by digesting anti-DYKDDDDK antibody, and validated its functionality as described in Morisaki et al (Science 2016).

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s): U-2 OS cells from ATCC

Authentication: U-2 OS cells were authenticated by STR profiling (ATCC) and morphological assessments.

Mycoplasma contamination: n We confirmed that all cell lines tested negative for mycoplasma contamination.

Commonly misidentified lines (See ICLAC register): No commonly misidentified lines were used.

Palaeontology

Specimen provenance: *Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).*

Specimen deposition: *Indicate where the specimens have been deposited to permit free access by other researchers.*

Dating methods: *If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.*

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals: *For laboratory animals, report species, strain, sex and age OR state that the study did not involve laboratory animals.*

Wild animals: *Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.*

Field-collected samples: *For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.*

Ethics oversight: *Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not.*

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

Human research participants

Policy information about studies involving human research participants

Population characteristics	*Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."*
Recruitment	*Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.*
Ethics oversight	*Identify the organization(s) that approved the study protocol.*

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

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	*Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.*
Study protocol	*Note where the full trial protocol can be accessed OR if not available, explain why.*
Data collection	*Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.*
Outcomes	*Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.*