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Reporting Summary

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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	Data was collected on a Vutara 352 System (Bruker 2020) using Vutara's SRX software 6.04.14.
Data analysis	For figure 1b, we used Fiji (ImageJ 1.52t, GNU General Public License) to cut out a selection from the raw data. For Figure 1c, 1d, 2a and c, Picasso software has been used (Version 0.2.8, https://github.com/jungmannlab/picasso). The localization densities in Figure 1e are calculated by the Vutara SRX software 6.04.14. Figure 1e, 2b and Extended data Figures 1, 2 and 3 were prepared with OriginPro 2017G 64-Bit. Figure 2d and Extended data Figure 5 were analyzed with Vutara SRX software 6.04.14 (Bruker 2020).

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

A reporting summary for this Article is available as Extended data file. Statistical source data are provided with this paper. The microscopy data that support the findings of this study will be available from the corresponding author upon reasonable request due to storage limitations.

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	In vitro hybridization assay data (Fig 1b and Extended data Fig. 1) were required from three independent measurements in triple, resulting in 1000s of datapoints per measurement. Microtubules were visualized in 5 and 4 cells for L-DNA-PAINT and R-DNA-PAINT (Fig 1c), giving an average microtubule diameter of 39 and 33 nm, of 22 and 9 microtubule regions respectively, which corresponds well to previous reports. In Figure 1e, the localization densities of 48 cells for P3 and LP3 and 20 cells for P13 and LP13 are compared. According to a paired T-test calculated by Origin Pro, the difference in mean value was significant. BrdU stainings were detected for 11 cells, which all showed an enrichment in R-DNA localizations in comparison to the L-DNA localizations. To show the multiplexing ability, we performed Ki67 and PCNA detection in the same cell for 3 cells, all showing discrete stainings as was published before. To illustrate the strength of L-DNA-PAINT, we show one FISH staining which shows the two integration sites of the HHV in the HEK293T genome in Figure 2d and one more in Extended data Fig. 5.
Data exclusions	No data was excluded.
Replication	In vitro hybridization assay (Fig 1b and Extended Fig. 1) was performed in triplet, the hybridization kinetics of L- and R-DNA were reproducible during those 3 experiments. Microtubules were visualized in 5 and 4 cells for L-DNA-PAINT and R-DNA-PAINT, respectively (Fig 1c), yielding a comparable microtubule diameter and Fourier ring correlation. For the hybridization of imagers in the nucleus, we measured 48 cells in 10 independent experiments for P3 and LP3 and 20 cells in 2 independent measurements for P13 and LP13 (Fig. 1e). We performed BrdU stainings on 11 cells in total, all of them showed the significant higher localization density for R-DNA-PAINT in the nucleus. The Ki67 and PCNA stainings in Hela cells were performed in triple, all of them allowed us to visualize the discrete stainings for those targets. The FISH experiments were undertaken for 30 cells, of which we could detect the two integration sites only twice due to the spatial separation of the integration sites and as such a low probability to detect both of them in the same image plane.
Randomization	For all experiments, despite Figure 1c, we performed interdependent experiments on the same cells (since we performed both left- and right-handed PAINT experiments on the same cell). As such, samples intrinsically were not allocated in separated groups.
Blinding	Localization densities were determined by the Vutara SRX software and as such we renounced from blinding.

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	Monoclonal Mouse anti-alpha-tubulin (Clone B-5-1-2, Sigma-Aldrich, T5168), Polyclonal Goat biotinylated anti-mouse antibody (BD Biosciences, Pharmingen, Catalog Nr. 550337), Biotinylated Anti-BrdU antibody (Clone BU20A, only available in the eBiosciences BrdU kit for IHC/ICC Colorimetric, Invitrogen, Thermo Scientific, Cat Nr. 8800-6599), Purified Mouse Anti-Ki67 (Clone B56, BD Biosciences, Pharmingen, Cat Nr. 556003) and donkey anti-mouse antibody (Jackson ImmunoResearch Europe Ltd, Cat. Nr. 715-005-150) were used in this study.
Validation	Anti-tubulin was validated by Sigma-Aldrich for indirect immunofluorescence on cultured human and chicken fibroblasts and have been used by our laboratory for super-resolution microscopy on Ptk2 cells (Ries et al. Nature Methods 2012). Biotinylated anti-mouse antibody was tested for ELISA and frozen, paraffin, and zinc-fixed immunohistochemistry by BD Pharmingen TM. Anti-Ki67 antibodies are regularly tested for intracellular staining and bioimaging of Hela, A549 and U2OS cells by BD Pharmingen

TM. The Anti-BrdU BU20A antibody has been tested by intracellular staining and flow cytometric analysis of BrdU-labeled mouse splenocytes by eBiosciences.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	HeLa ATCC CCL2 and 293T ATCC CRL-3216
Authentication	The Hela cell lines used were not authenticated, the epithelial phenotype was confirmed for the 293T cells.
Mycoplasma contamination	The cells used in this study were tested negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	None used in this study