Single-molecule imaging reveals RNA polymerase II dynamics and TAF1-dependent promoter-proximal pause release

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Supplementary Information

SNAP-*POLR2A* HDR Repair template sequence (5' to 3')

GCTCAGAAGCGCCGAGAGCGCGGCCGGGACGGTTGGAGAAGAAGGCGGCTCCCGGAAGGGGGAGAGACAAACTGC
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TAF1-Halo HDR Repair template sequence (5' to 3')

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AAAAGAGCAAGCTCAT

Supplementary Table 1: Homology-directed repair templates for CRISPR-Cas9 knock-in. Template sequences are annotated as follows: nucleotides highlighted in green indicate the

upstream homology arm; purple bold letters denote the SNAP or HaloTag coding sequence; nucleotides highlighted in yellow indicate the downstream homology arm; green bold letters mark the start codon; and red bold letters indicate the stop codon. The top sequence corresponds to the SNAP-tag knock-in at the *POLR2A* locus, and the bottom sequence corresponds to the HaloTag knock-in at the *TAF1* locus.

	Sequence (5' to 3')
POLR2A crRNA	GCCACCCCCGTGCATGGCGG
TAF1 crRNA	GACTTGGACTCTGATGAATG
PCNA crRNA	CCACTCCGCCACCATGTTCG

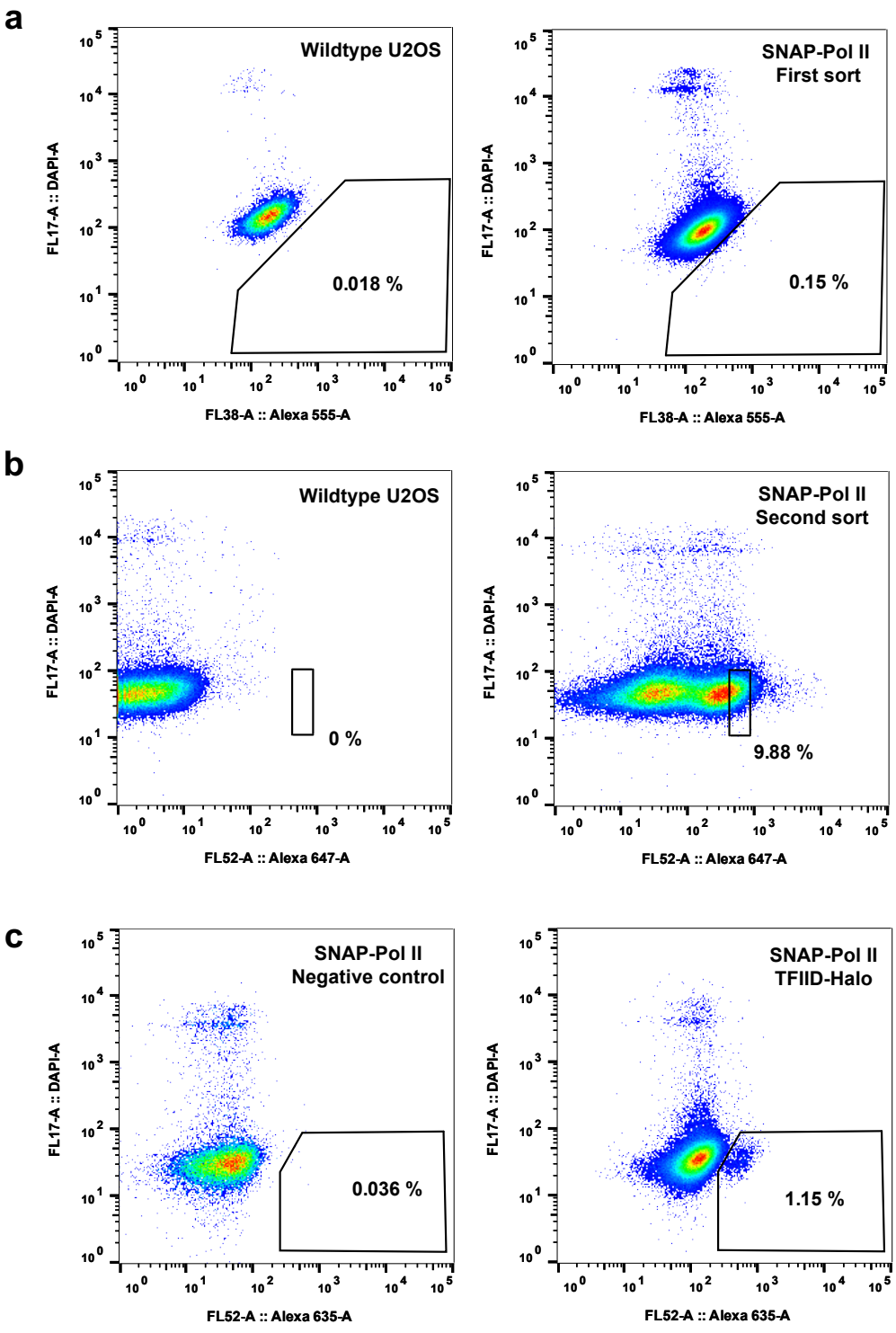
Supplementary Table 2: crRNA sequences targeting human *POLR2A*, *TAF1*, and *PCNA* genes.

Halo-PCNA HDR Repair template sequence (5’ to 3’)
TCACTTTCACTTTCGTGGCTGGAAACTTTCTACCCACGTAGTGGGAGGCTGAGGAGCCACCATAAAGCTGGGGCT TGACGAGCCGGGACCGGGACCCGATCTCCACATATGCCCGGACTTGTTCTGCGGCCGGGTTCAGGAGTCAAAGAG GCGGGGAGACCTGCGCGACGCTGCCCCGCCCTGCGCCCGCTTCCTCCAATGTATGCTCTAGGGGGCGGGCCTCGC GGGGAGCATGGACACGATTGGCCCTAAAGTCTTCCCCGCAAGGCCGTGGGCTGGACAGCGTGGTGACGTCGCAAC GCGGCGCAGGGTGAGAGCGCGCGCTTGCGGACGCGGCGGCATTAAACGGTTGCAGGCGTAGCAGAGTGGTCGTTG TCTTTCTAGGTCTCAGCCGGTCGTCGCGACGTTCGCCCGCTCGCTCTGAGGCTCCTGAAGCCGAAACCAGCTAGA CTTTCCTCCTTCCCGCCTGCCTGTAGCGGCGTTGTTGCCACTCCGCCACCATGGCAGAAATCGGTACTGGCTTTC CATTCGACCCCCATTATGTGGAAGTCCTGGGCGAGCGCATGCACTACGTCGATGTTGGTCCGCGCGATGGCACCC CTGTGCTGTTCCTGCACGGTAACCCGACCTCCTCCTACGTGTGGCGCAACATCATCCCGCATGTTGCACCGACCC ATCGCTGCATTGCTCCAGACCTGATCGGTATGGGCAAATCCGACAAACCAGACCTGGGTTATTTCTTCGACGACC ACGTCCGCTTCATGGATGCCTTCATCGAAGCCCTGGGTCTGGAAGAGGTCGTCCTGGTCATTCACGACTGGGGCT CCGCTCTGGGTTTCCACTGGGCCAAGCGCAATCCAGAGCGCGTCAAAGGTATTGCATTTATGGAGTTCATCCGCC CTATCCCGACCTGGGACGAATGGCCAGAATTTGCCCGCGAGACCTTCCAGGCCTTCCGCACCACCGACGTCGGCC GCAAGCTGATCATCGATCAGAACGTTTTTATCGAGGGTACGCTGCCGATGGGTGTCGTCCGCCCGCTGACTGAAG TCGAGATGGACCATTACCGCGAGCCGTTCCTGAATCCTGTTGACCGCGAGCCACTGTGGCGCTTCCCAAACGAGC TGCCAATCGCCGGTGAGCCAGCGAACATCGTCGCGCTGGTCGAAGAATACATGGACTGGCTGCACCAGTCCCCTG TCCCGAAGCTGCTGTTCTGGGGCACCCCAGGCGTTCTGATCCCACCGGCCGAAGCCGCTCGCCTGGCCAAAAGCC TGCCTAACTGCAAGGCTGTGGACATCGGCCCGGGTCTGAATCTGCTGCAAGAAGACAACCCGGACCTGATCGGCA GCGAGATCGCGCGCTGGCTGTCGACGCTCGAGATTTCCGGCTCCGGACTCAGATCTCGAGCTCAAGCTTCGAATT CTGCAGTCGACGGTACCGCGGGCCCGGGATCCACCGGATCTAGCTTCGAGGCGCGCCTGGTCCAGGGCTCCATCC TCAAGAAGGTGTTGGAGGCACTCAAGGACCTCATCAACGAGGCCTGCTGGGATATTAGCTCCAGCGGTGTAAACC TGCAGAGCATGGACTCGTCCCACGTCTCTTTGGTGCAGCTCACCCTGCGGTCTGAGGGCTTCGACACCTACCGCT GCGACCGCAACCTGGCCATGGGCGTGAACCTCACCAGGTGAGCCTCGCGGCCCCGGGAAGCCGGCCCCGGCCCGC CTGCACCTCCGGTGCTTGGCGGGAGCGCTTTCGAGCCTAGCCCTCATTGGCTGGCGTGGGCCATCCGCGCCTTCT CATTGGCCTGCCACGCAGTGGGGTGGGGCCCAGCTGAGCGCGCGGTTCGGAAAAGCCCGCGCTGGCTGCTGCGCG AACCTGCTTTTTCGCGCCAAAGTCACAAAGCGGGTGGTGGCGGGAAAATCAAGGGTTTTTCCGCAGTGCCAGGAA CACTGTTCCAGGGCTCTTT

Supplementary Table 3: Homology-directed repair templates for CRISPR-Cas9 knock-in**.** Annotations follow **Supplementary Table 1**. The sequence corresponds to the Halo-tag knock-in at the N-terminus of the *PCNA* locus.

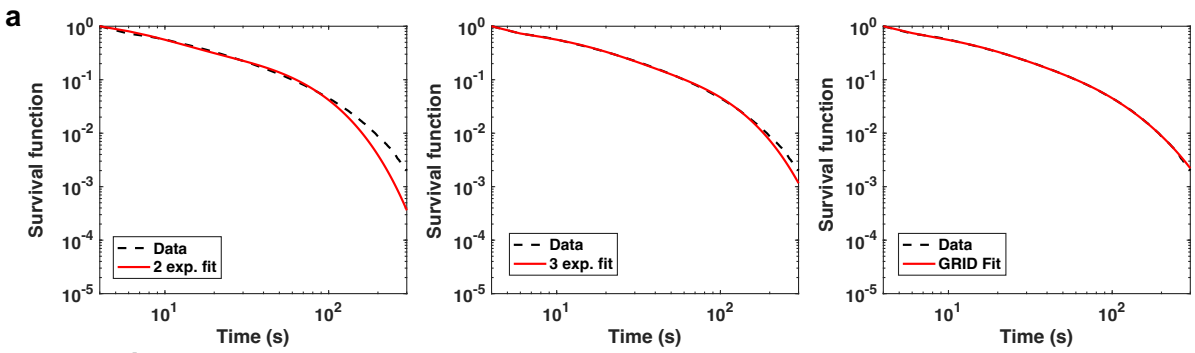
	Sequence (5’ to 3’)
SNAP-POLR2A forward primer (5’ phosphorylated)	AAGGGGGAGAGACAAACTGC
SNAP-POLR2A reverse primer	ACAGAAGAGGAGGAAGCTACC
TAF1-Halo forward primer	GTCCGAGCTTGAGAGATGAC
TAF1-Halo reverse primer (5’ phosphorylated)	GCTTGCTCTTTTCCGTAGTG
Halo-PCNA forward primer	CTGAGGAGCCACCATAAAGC
Halo-PCNA reverse primer (5’ phosphorylated)	CACCCGCTTTGTGACTTTGG

Supplementary Table 4: PCR primers to amplify homology-directed repair templates**.**



Supplementary Figure 1: FACS isolation of SNAP-*POLR2A TAF1*-Halo dual knock-in cell lines. Cells electroporated with gene-specific sgRNA constructs and SNAP–*POLR2A* or *TAF1*–Halo repair templates were sorted using FACS to isolate fluorescently labeled cells. For all panels, the Y-axis represents DAPI fluorescence (FL17-A:: DAPI-A) used to exclude dead cells. For all

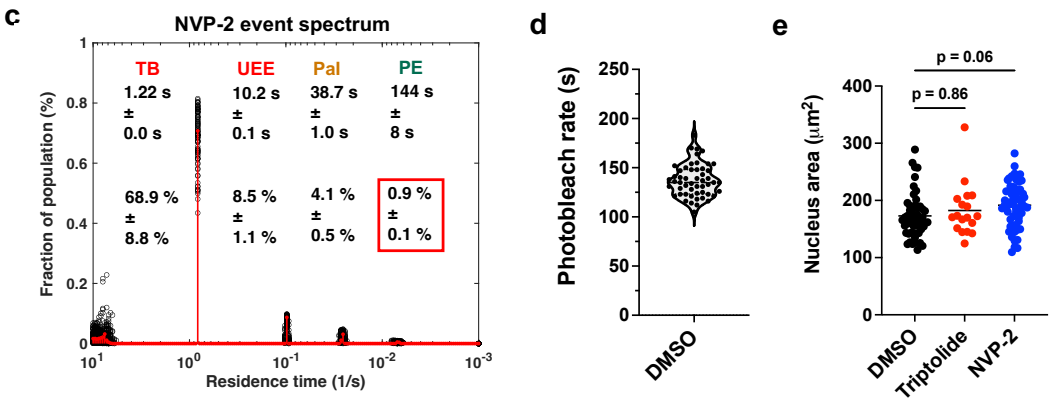
panels, the black box within the FACS plot indicates the gate used for cell sorting. **a,** Dot plots show SNAP-Cell TMR-549 fluorescence (FL38-A:: Alexa 555-A) for wild-type U2OS cells (left) and SNAP–*POLR2A* knock-in cells (right). A sorting threshold was set based on the maximum intensity detected in wild-type cells. 0.15% fluorescent cells (n = 774) above this threshold were collected for expansion. **B,** Dot plots show SNAP-Cell SiR-647 fluorescence (FL52-A:: Alexa 647-A) for wild-type U2OS cells (left) and SNAP–*POLR2A* knock-in cells sorted from panel a (right). 9.88 % fluorescent cells (n = 207,090) above the wild-type threshold were collected to isolate homozygous integration of the SNAP-tag. **c,** Dot plots show HaloTag JF-635 fluorescence (FL52-A:: Alexa 635-A) for SNAP–*POLR2A* knock-in cells (left) and dual SNAP–*POLR2A* / *TAF1*–Halo knock-in cells (right). 1.15 % fluorescent cells (n = 16,724) above the SNAP–*POLR2A* background threshold were collected to establish the dual knock-in line.



b

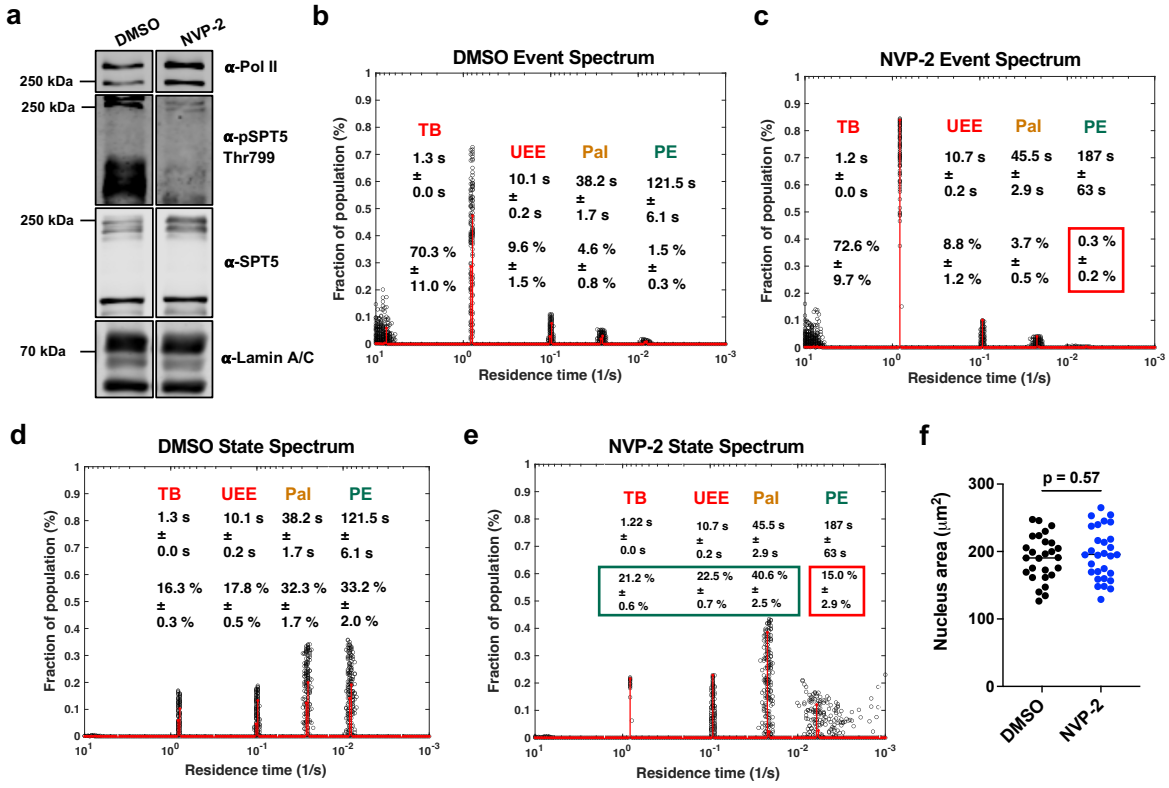
Event spectrum	P_1 (%)	τ_1 (s)	P_2 (%)	τ_2 (s)	P_3 (%)	τ_3 (s)	P_4 (%)	τ_4 (s)
DMSO (n = 23)	65.8 ± 10.2	1.23 ± 0.01	8.0 ± 1.4	10.50 ± 0.24	3.2 ± 0.5	37.0 ± 2.9	1.8 ± 0.4	115.6 ± 8.1
Triptolide (n = 18)	69.7 ± 11.7	1.22 ± 0.01	8.8 ± 1.5	10.37 ± 0.23	3.4 ± 0.6	41.1 ± 3.9	1.2 ± 0.4	132.7 ± 18.3

State spectrum	P_1 (%)	τ_1 (s)	P_2 (%)	τ_2 (s)	P_3 (%)	τ_3 (s)	P_4 (%)	τ_4 (s)
DMSO (n = 23)	16.1 ± 0.4	1.23 ± 0.01	16.6 ± 0.7	10.50 ± 0.24	23.4 ± 2.5	37.0 ± 2.9	41.3 ± 2.5	116.4 ± 8.0
Triptolide (n = 18)	17.4 ± 0.3	1.22 ± 0.01	18.7 ± 0.8	10.37 ± 0.23	29.2 ± 3.7	41.1 ± 3.9	32.9 ± 3.8	134.6 ± 16.5



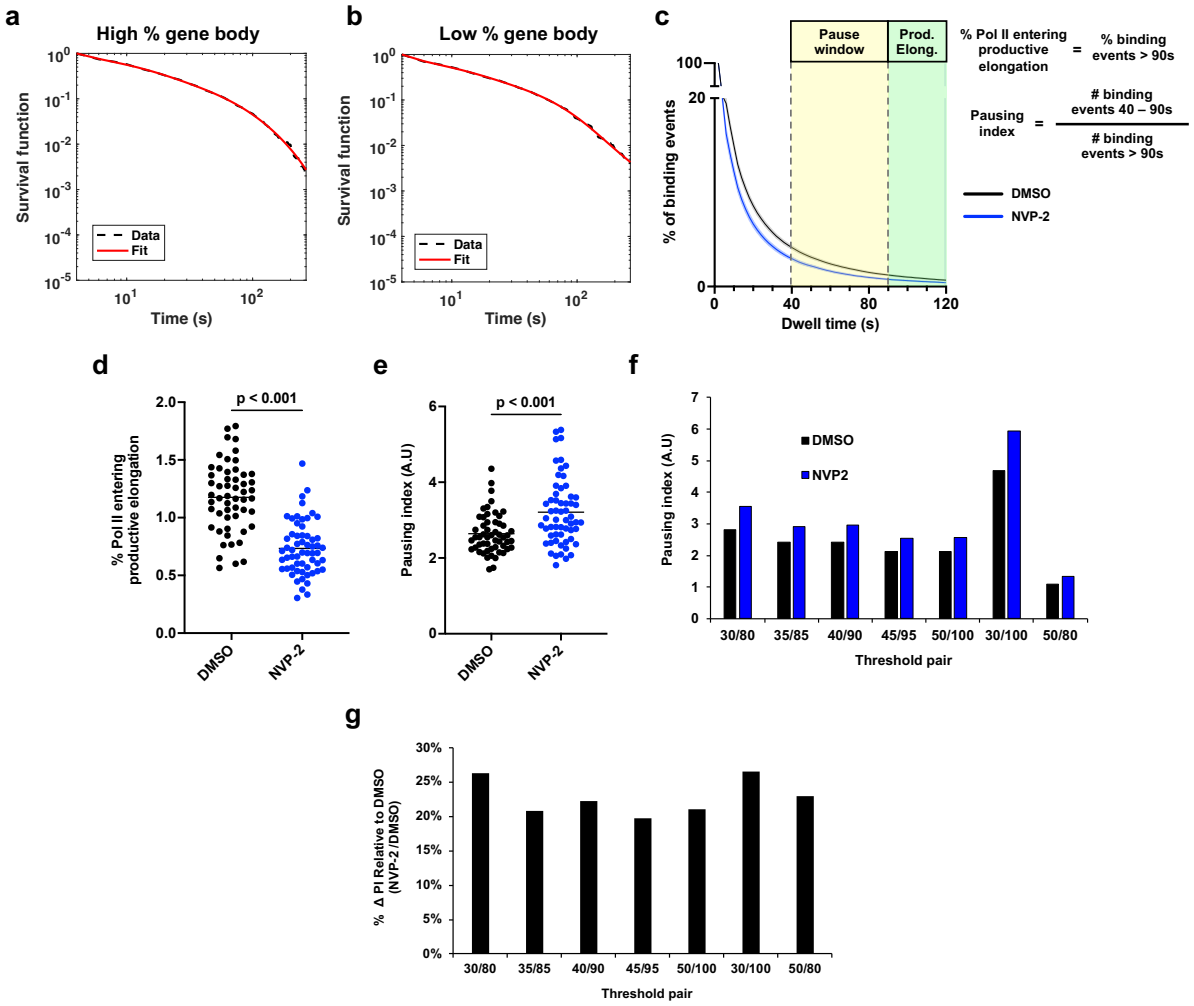
Supplementary Figure 2: Transcription inhibition defines GRID populations. a, Dwell-time distribution (black dashed line) and corresponding multi-exponential fits (red line) using two (left), three (middle), and four exponentials (GRID, right) for DMSO-treated cells. **b,** GRID event (top) and state (bottom) spectra data of Pol II chromatin binding events in DMSO (n = 23 cells, 30-min) and Triptolide (n = 18 cells, 500 nM, 30-min) treated cells. P_1 represents the fraction of Pol II binding events in the most transient, short-lived population, while τ_1 corresponds with the residence time for that population. P_4 and τ_4 are the most stable, long-lived fractions. Boxes shaded in red are percent populations/residence times that consistently decrease and are outside the error range of DMSO, while boxes shaded in green increase. **c,** GRID event spectra for NVP-2 treated cells (n = 59 cells, 500 nM, 30-min). **d,** Distribution of photobleaching rates in DMSO-

treated cells (n = 52 cells, 30-min), determined from the total fluorescence decay within each nucleus. **e,** Nuclear area per cell in DMSO (n = 52 cells), Triptolide (n = 18 cells), and NVP-2 (n = 59 cells) conditions. Each point represents one nucleus. A One-Way ANOVA with Sidak's multiple comparisons test was performed to determine statistical significance. Drug concentration and treatment time indicated in **b** and **c**. Source data are provided in the Source Data file.



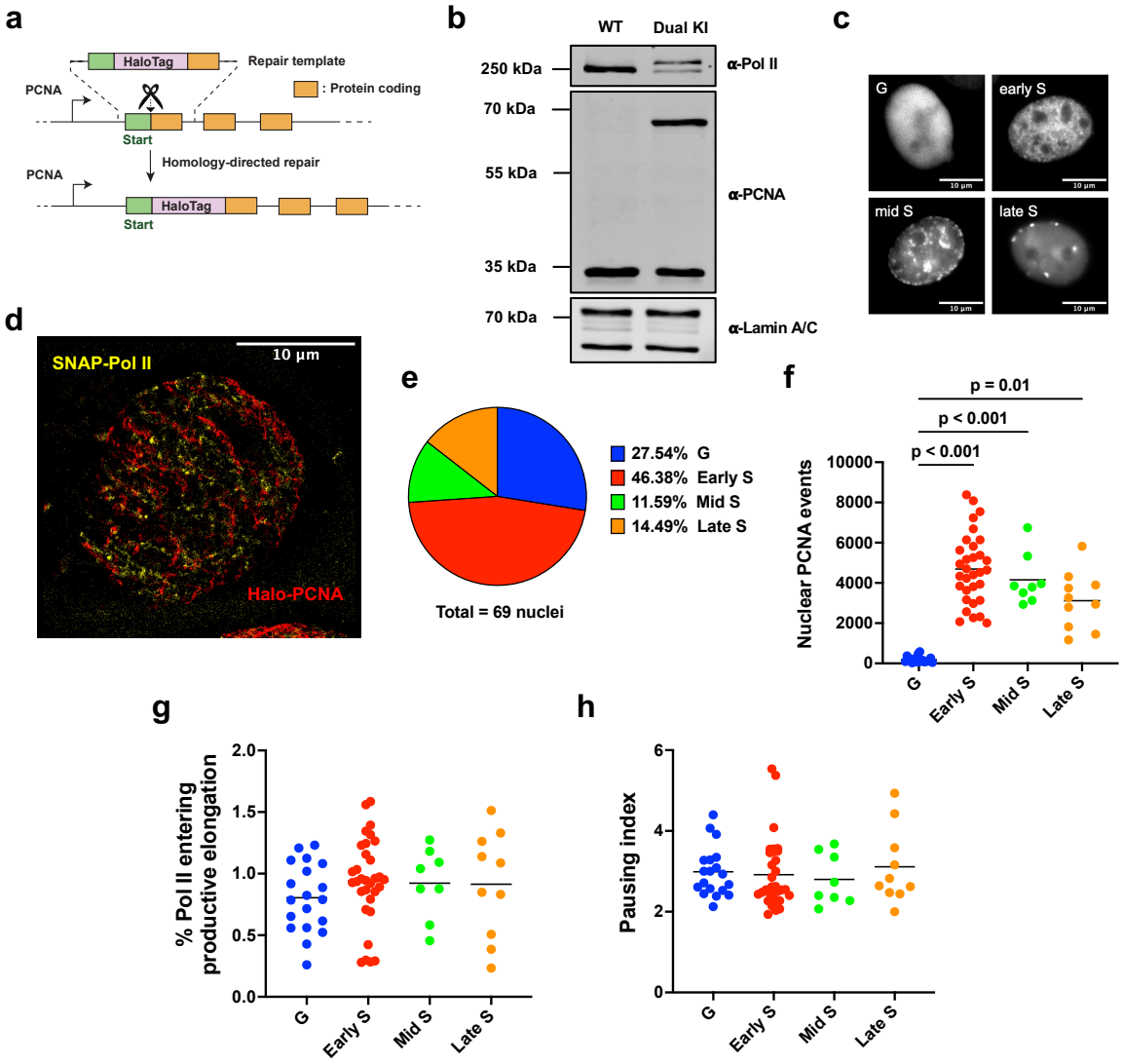
Supplementary Figure 3: 2-hour NVP-2 treatment displays similar shifts in GRID spectra as 30-min treatment. a, Western blot analysis demonstrating transcription inhibition with NVP-2. Nuclear extracts from dual knock-in (SNAP-Pol II TAF1-Halo) cell lines were treated with DMSO and 50 nM NVP-2 for 2-hours and probed with antibodies against Pol II, phospho-SPT5 (Thr799), total SPT5, and Lamin A/C (loading control). **b–e,** GRID event (**b, c**) and state (**d, e**) spectra for DMSO (n = 27 cells) and NVP-2 (n = 29 cells, 50 nM) treated cells. Boxes shaded in red are percent populations that consistently decrease and are outside the error range of DMSO while boxes shaded in green increase. **f,** Nuclear area in DMSO (n = 27 cells) and NVP-2 (n = 29 cells) treated conditions. Each point represents data points from a single nucleus. A two-

tailed unpaired t test was performed to determine statistical significance. Source data are provided in the Source Data file.



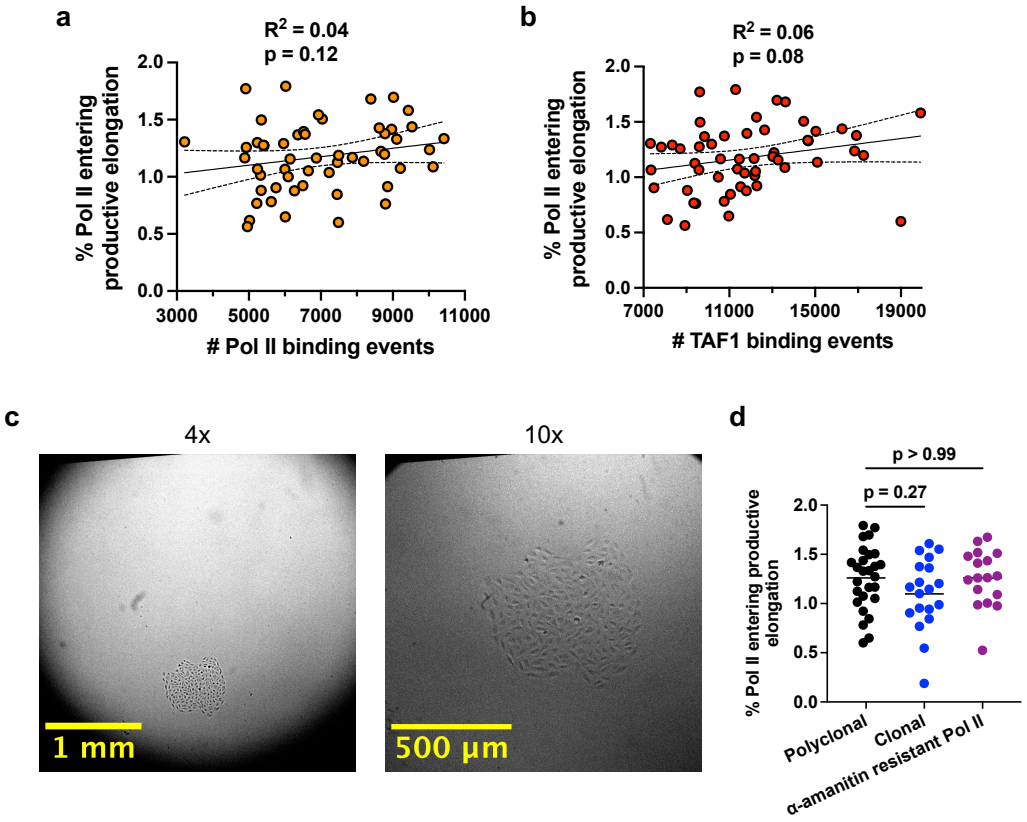
Supplementary Figure 4: Single cell transcription metrics and pausing index. a-b, Dwell-time distribution (black dashed line) and corresponding multi-exponential GRID fit (red) for the cells in Fig. 3b. (**a**) and Fig. 3c (**b**). **c,** Survival curve of Pol II binding events in DMSO (n = 54, 2-hours treatment) and NVP-2 (n = 59, 50 nM, 2-hours) treated cells, represented as a percentage of total. Survival curve is truncated at 120s for display. **d-e,** Per-cell percentages of Pol II binding events reaching the productive elongation state (**d**) and pausing index **(e**) for DMSO (n = 54 cells) and NVP-2 (n = 59 cells) treated cells. Drug concentration and treatment time indicated in **c**. Each dot represents data for a single cell. A two-sided Mann-Whitney U test was performed to

determine statistical significance. For **d**, p-value = 6×10^{-14}. For **e**, p-value = 1×10^{-4}. **f,** Paused/Productive Elongation dwell-time threshold pairs and their corresponding pausing index in DMSO and NVP-2 treated cells in **c**. **g,** Percent increase of pausing index in NVP-2–treated cells relative to DMSO (NVP-2 / DMSO) for each threshold pair shown in **f**. Bar charts represent a single pausing index value derived from aggregated data in all cells within each condition. Source data are provided in the Source Data file.



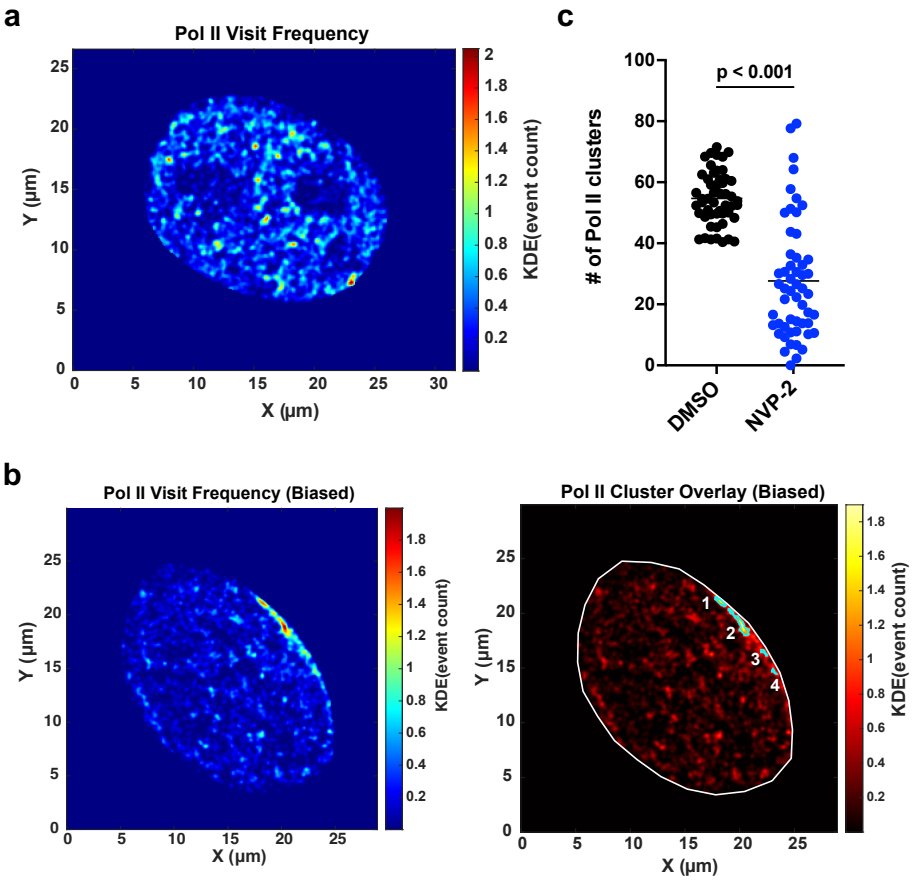
Supplementary Figure 5: Single cell heterogeneity in Pol II reaching productive elongation is not due to the cell cycle. a, CRISPR-Cas9 strategy to knock in an N-terminal HaloTag at the endogenous PCNA locus in the SNAP-tagged Pol II lines. **b,** Immunoblot confirming expression of tagged proteins. This experiment was performed once. Nuclear extracts from WT and dual

knock-in (SNAP-Pol II / Halo-PCNA) lines were probed for PCNA, Pol II, and Lamin A/C (loading control). **c,** Raw single-frame images of Halo-PCNA showing four characteristic patterns used to assign cell state: G (G1 or G2, diffuse signal), early S (numerous heterogenous distributions of puncta), mid S (numerous large peripheral puncta), and late S (very few large puncta). **d,** Dual-color live cell image of SNAP-Pol II (yellow) and Halo-tagged PCNA (red) in the same nucleus (max intensity projection). **e,** Parts of whole summary of cell cycle states that were imaged (n = 69 cells). **f,** Per-cell nuclear PCNA event counts in G (n = 19), early S (n = 32 cells, p-value = 4×10^{-10}), mid S (n = 8 cells, p-value = 5×10^{-4}), and late S (n = 10 cells). A Kruskal-Wallis with Dunn's post-hoc was performed to determine statistical significance. **g-h,** Per-cell percentages of Pol II binding events with dwell time >90 s (**g**), and pausing index (**h**) from cells analyzed in **f**. Statistics are performed as in **f**. The absence of a p-value indicates a p-value that is > 0.05. Source data are provided in the Source Data file.



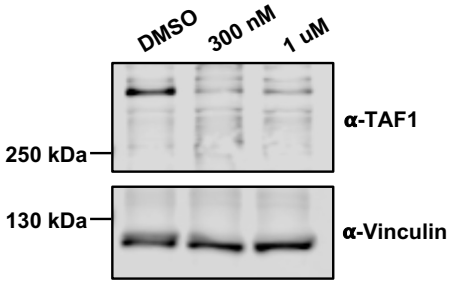
Supplementary Figure 6: Single cell heterogeneity in Pol II entering productive elongation is not due to protein levels or genetic variability from polyclonal cells. a-b, Linear correlation plot of the number of Pol II (Orange, **a**) and TAF1 (Red, **b**) nuclear binding events plotted as a function of the percentage of Pol II reaching productive elongation. The solid

black line indicates the best-fit line from a simple linear regression, and the black dotted line indicates the 95% confidence intervals. n = 54 total cells analyzed under 2-hour DMSO condition. **c,** Inverted brightfield images of a colony derived from limiting dilution in a 96-well imaging dish, acquired at 4x (left) and 10x (right) magnification. Cells were plated at 0.3 cells per well. **d,** Per-cell percentages of Pol II entering productive elongation in endogenous CRISPR-engineered U2OS SNAP-POLR2A TAF1-Halo polyclonal cells (n = 27 cells), single cell clone (n = 19 cells, from **c**), and U2OS cells expressing an alpha-amanitin resistant RPB1 expressed from a plasmid (n = 17 cells). All cells were treated with DMSO for 2 hours. A Kruskal-Wallis with Dunn's post-hoc was performed to determine statistical significance. Source data are provided in the Source Data file.

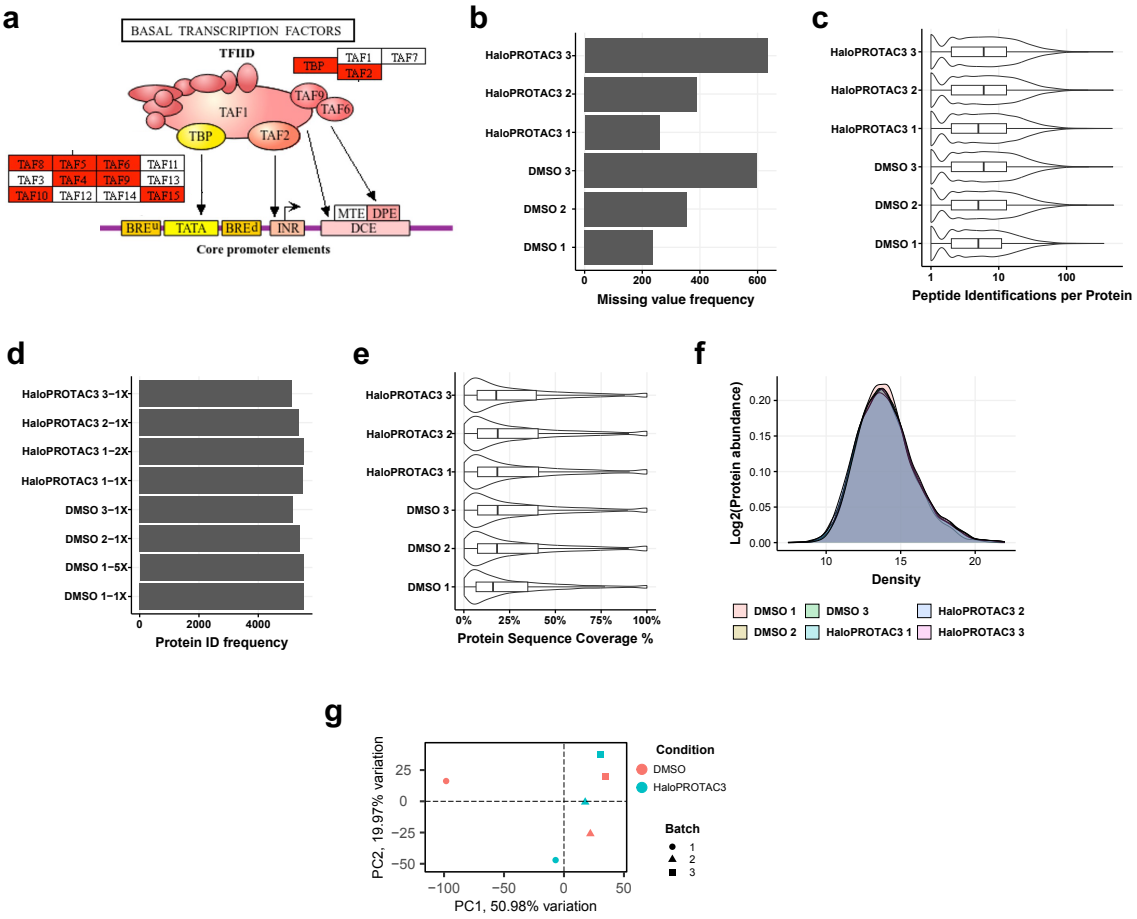


Supplementary Figure 7: Spatial heatmap analysis visit frequency and 2-hour drug treatment dataset. a, Visit frequency (Kernel density estimate map) of Pol II binding events in the nucleus in Fig. 4a. Warmer colors indicate higher revisit frequency. Scale is in microns and color bar reports KDE event density. **b,** Visit frequency (left) and cluster overlay (right) of Pol II

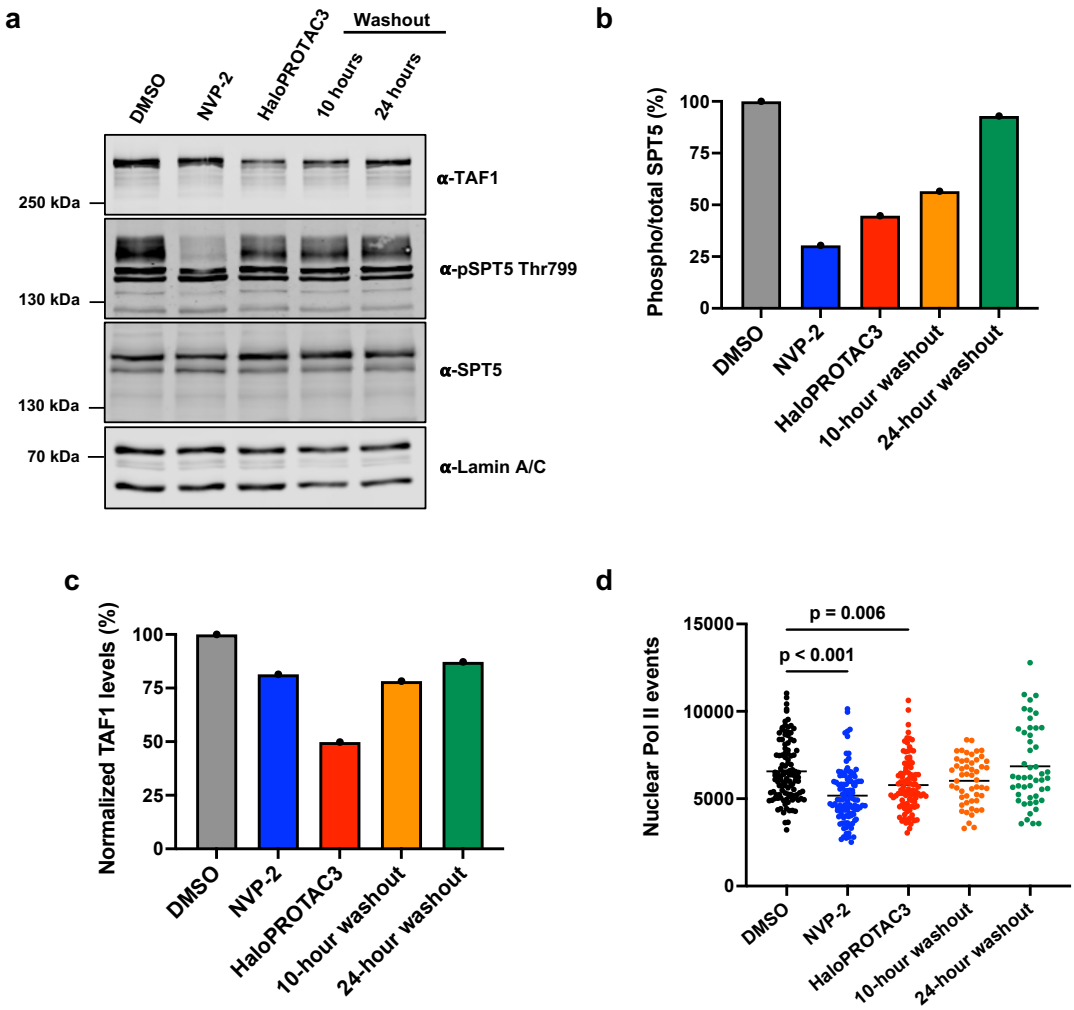
binding events in the nucleus of a cell excluded in the heatmap analysis due to abnormal and biased hotspot selection. **c,** Total number of Pol II clusters detected across single cells in DMSO (n = 51 cells) and NVP-2 (n = 54 cells) treated cells using the average KDE threshold across all DMSO-treated cells. A two-sided Mann-Whitney U test was performed to determine statistical significance. For NVP-2 vs. DMSO, p-value = 7×10^{-13}. Source data are provided in the Source Data file.



Supplementary Figure 8. Whole-cell immunoblot showing degradation of TAF1 with HaloPROTAC3. Dual knock-in (SNAP-Pol II TAF1-Halo) lines were treated for 2 hours with DMSO, 300 nM HaloPROTAC3, or 1 μM HaloPROTAC3. The effects of 300 nM HaloPROTAC3 for 2-hours on TAF1 levels are representative of four independent experiments which produced similar results. Source data are provided in the Source Data file.



Supplementary Figure 9: Mass spectrometry QC. U2OS dual knock-in cells were treated with DMSO or 300 nM HaloPROTAC3 for 2 h and analyzed by label-free quantitative mass spectrometry (DMSO and HaloPROTAC3, n = 3 biological replicates). **a,** Schematic of the KEGG basal transcription factor pathway with subunits detected by mass spectrometry highlighted in red (undetected peptides in white). Adapted with permission from KEGG pathway map hsa03022, Basal transcription factors, Kanehisa Laboratories[1]. **b,** Proportion of missing values per protein in each condition. **c,** Distribution of the number of unique peptides identified per protein. **d,** Frequency distribution of protein identifications across samples. **e,** Protein sequence coverage (%) across the dataset. **f,** Distribution of $\log_2$-transformed protein abundances, shown as density plots for each condition. **g**, Principal component analysis (PCA) of protein abundance profiles.



Supplementary Figure 10: TAF1 rescue phospho-SPT5 recovery and Pol II levels. a-c, Immunoblot (n = 1 independent experiment) measuring the recovery of TAF1 (**a, c**) and pSPT5 levels (**a, b**). Immunoblot signals were quantified using ImageStudio software. **d,** Total number of nuclear Pol II binding events detected in DMSO (n = 111 cells, 2 hours), NVP-2 (n = 110 cells, 50 nM, 2 hours), HaloPROTAC3 (n = 101 cells, 300 nM, 2 hours), 10-hour (n = 52 cells) and 24-hour (n = 49 cells) HaloPROTAC3 washout condition cells in Fig. 6e and 6f. For NVP-2 vs. DMSO, p-value = 6×10^{-9}. Source data are provided in the Source Data file.

References

1 Kanehisa, M., Furumichi, M., Sato, Y., Matsuura, Y. & Ishiguro-Watanabe, M. KEGG: biological systems database as a model of the real world. *Nucleic Acids Research* **53**, D672-D677 (2025). https://doi.org:10.1093/nar/gkae909