
The Wild West of spike-in normalization

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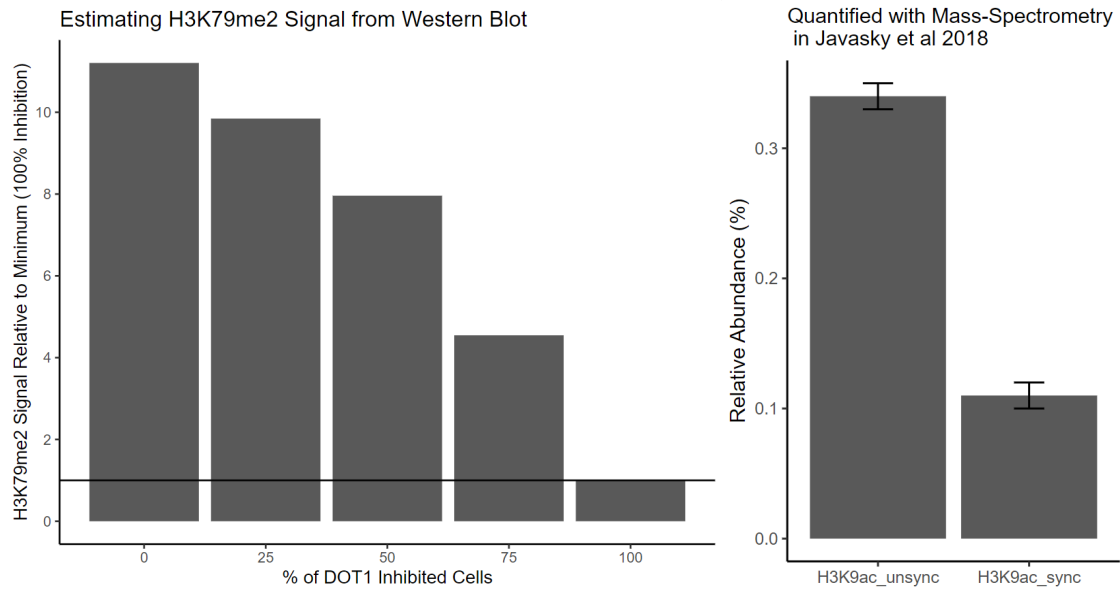
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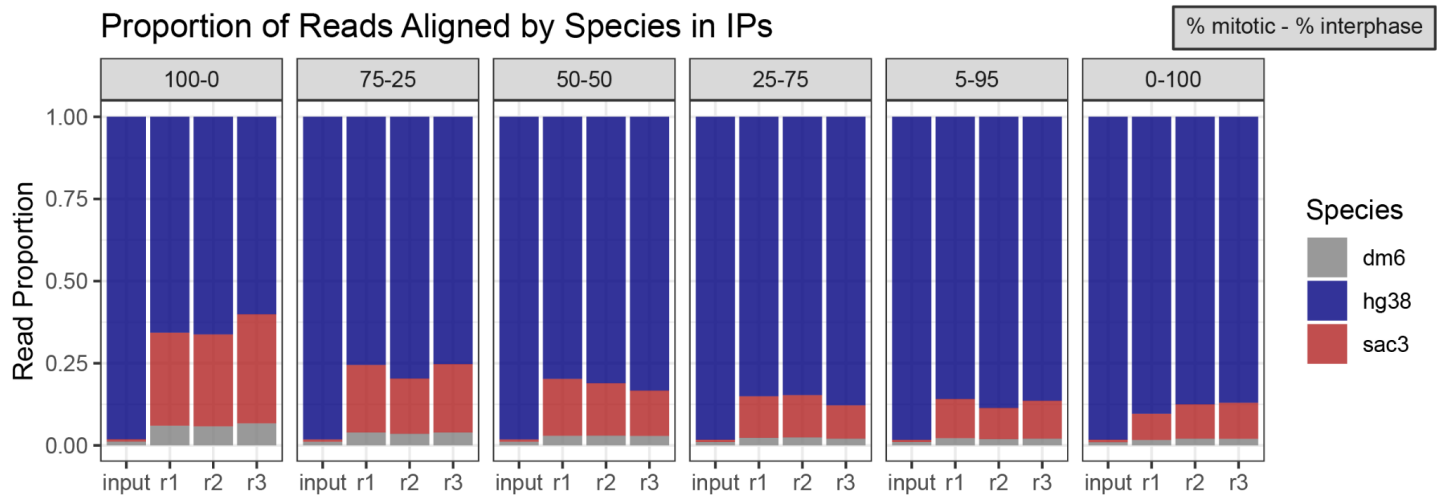
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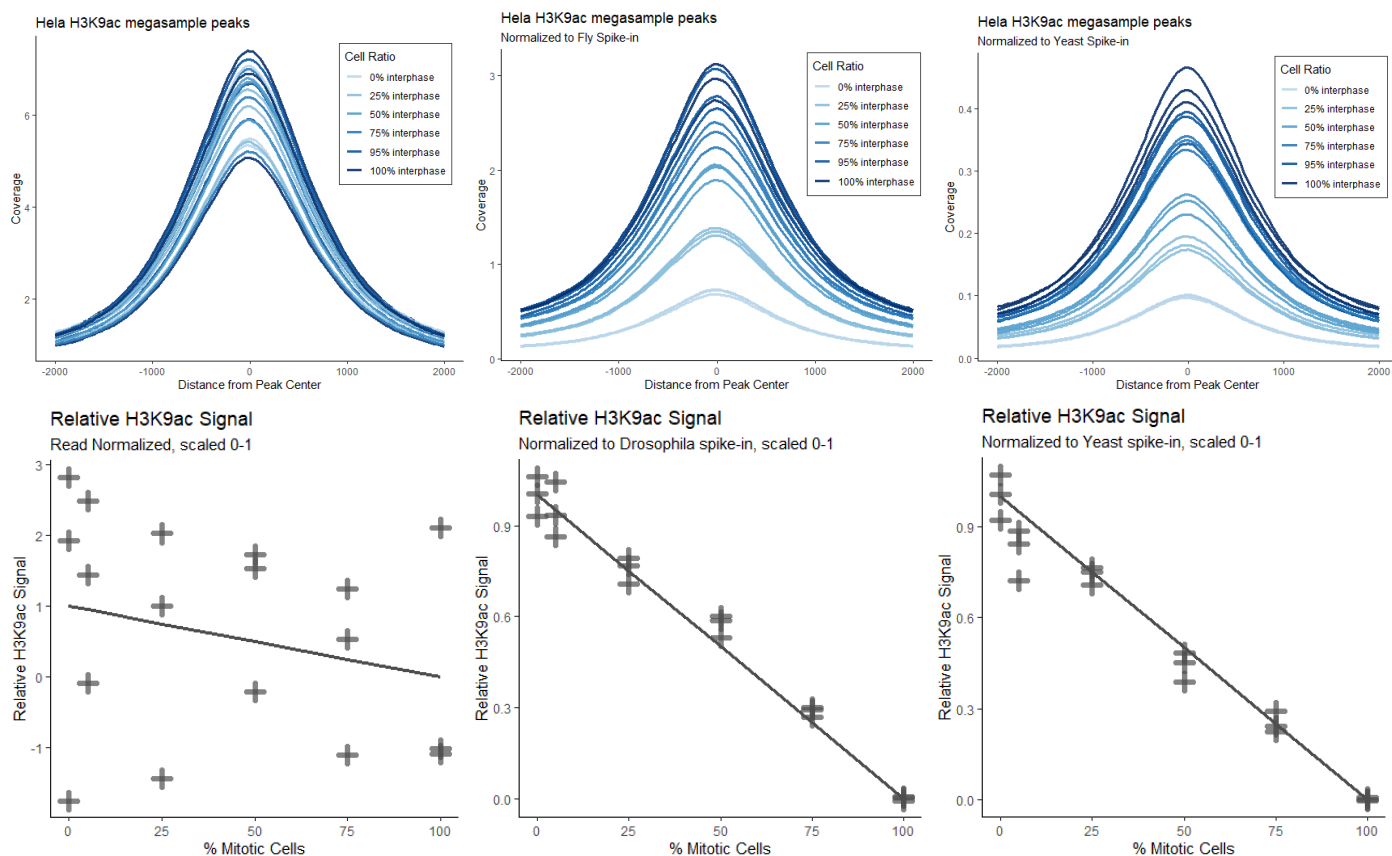
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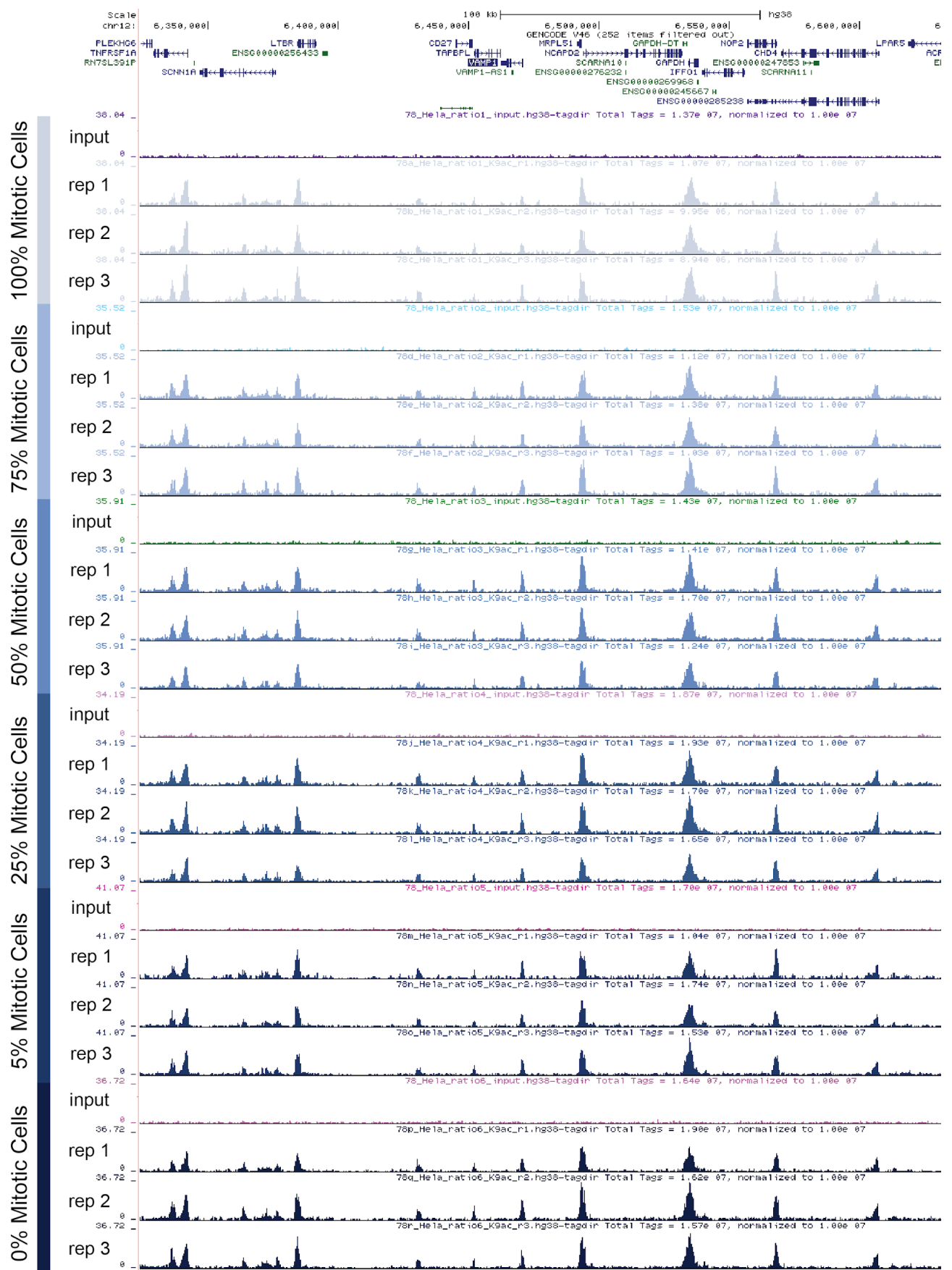
Supplemental Fig. 1. Estimation of dynamic range in EPZ5676-treated H3K79me2 titration (Orlando et al. generated) and H3K9ac titration datasets. **1a:** Signal estimated from Orlando et al. Fig. 1¹ with ImageJ and plotted as fold change relative to the minimum signal (in 100% DOT1 inhibited cells). **1b:** Signal estimated from Javasky et al. Fig 2b², where histone mark abundance was quantified with mass spectrometry.



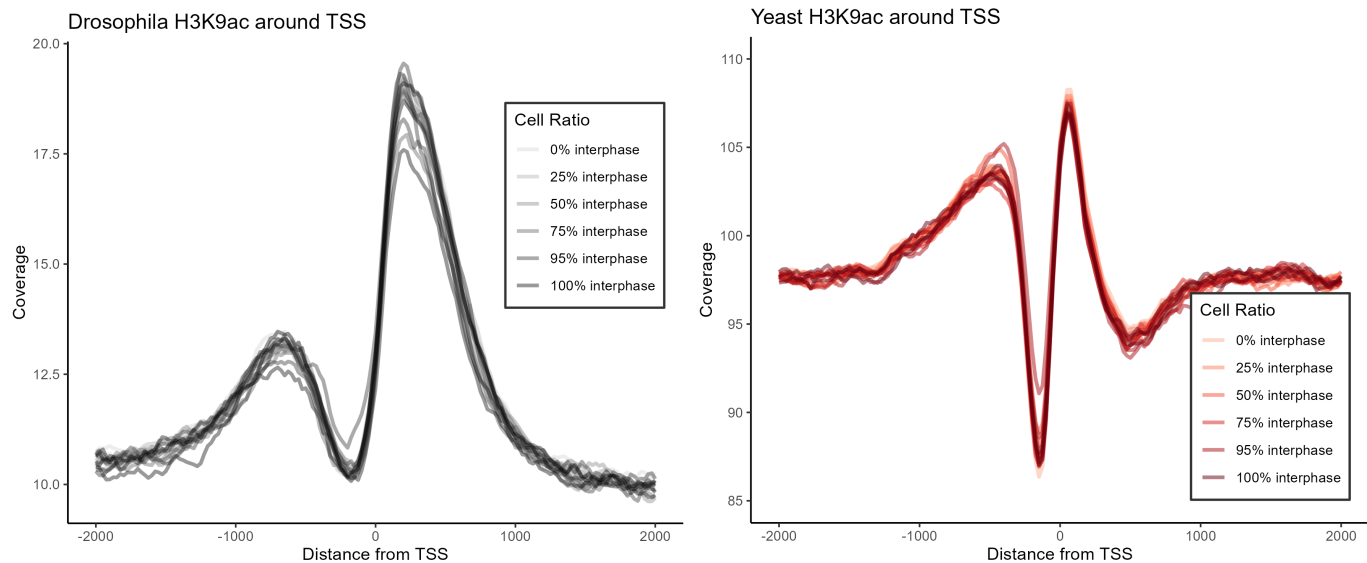
Supplemental Fig. 2. Proportion of reads aligning to each species (human, fly, yeast) in Mitotic/Interphase H3K9ac Titration experiment. All sequencing statistics and sample information available in **Supplemental Table 3**.



Supplemental Fig. 3. Histogram of H3K9ac signal at *Homo sapiens* TSSs, after spike-in normalization to *D. melanogaster* and *S. cerevisiae*. **Top:** Histogram of H3K9ac signal at all H3K9ac peaks. **Bottom:** Signal from histograms, quantified and plotted against line of expected signal. **Left:** read depth normalized signal; **middle,** normalized to *D. melanogaster* spike-in; **right,** normalized to *S. cerevisiae* spike-in. Histogram generated using HOMER annotatePeaks with parameters: tss hg38 -size 4000 -hist 25. Details on normalization in Methods and on Github at: https://github.com/lapatel22/spike_in_correspondence_2024/tree/main.

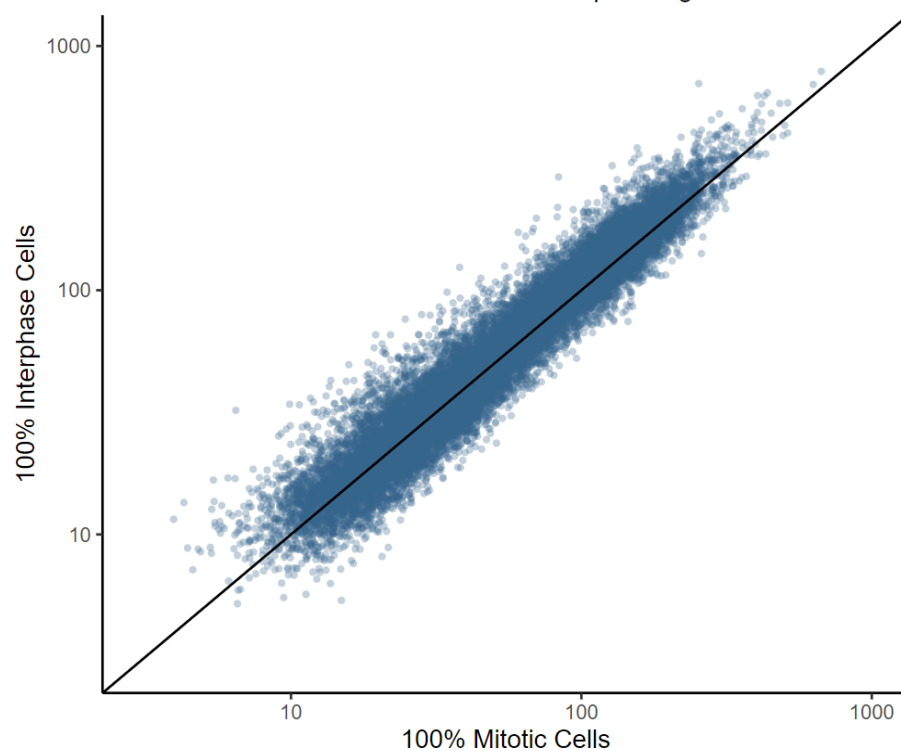


Supplemental Fig. 4. UCSC Genome Browser tracks of H3K9ac ChIP in HeLa-S3 Interphase and Mitotic titration experiment. Tracks are read-depth normalized, and each set of conditions is scaled to the same vertical axis. Note that replicates are technical, derived from the same pool of input chromatin.

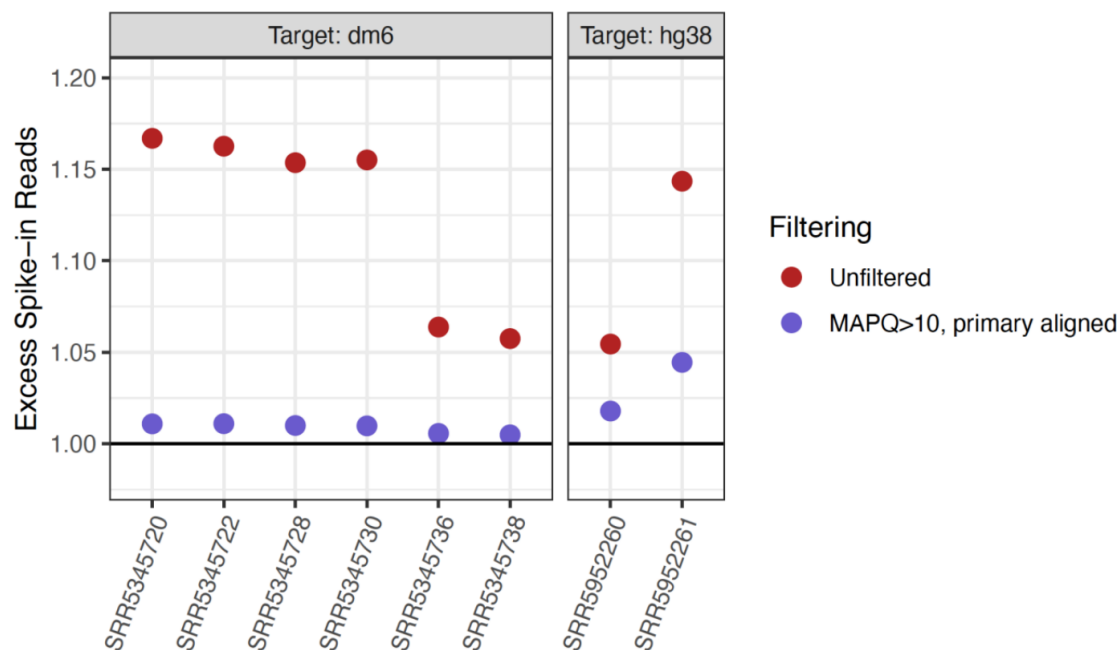


Supplemental Fig. 5. Histogram of read-depth normalized H3K9ac signal at *Drosophila melanogaster* (dm6) and *Saccharomyces cerevisiae* (sacCer3) TSSs. Histogram generated using HOMER annotatePeaks tss -size 4000 -hist 25.

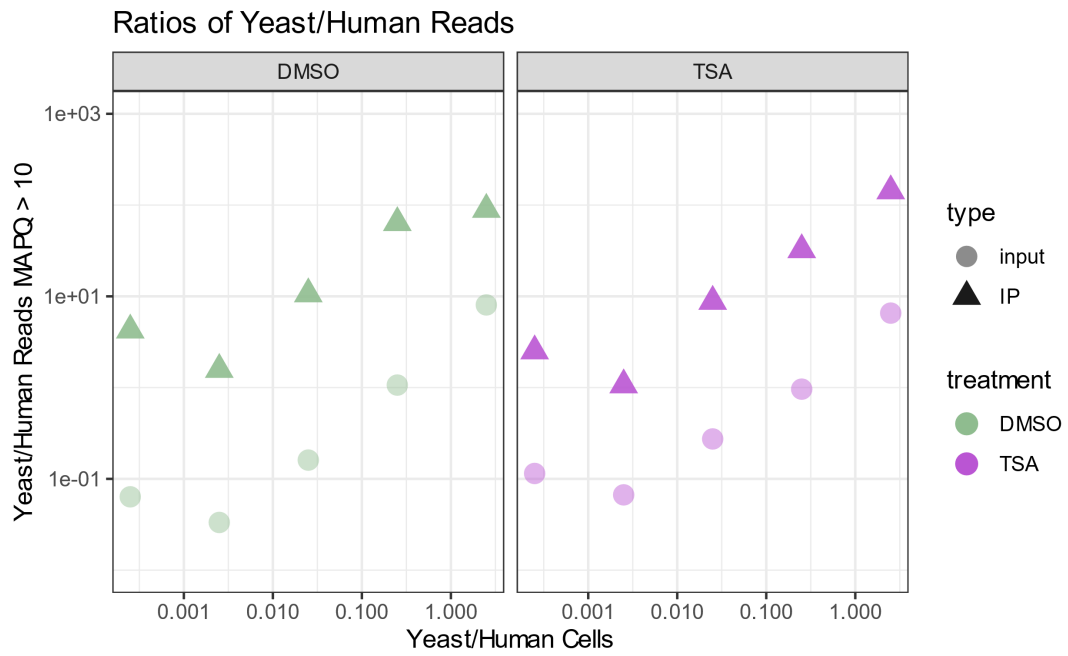
Comparing H3K9ac Signal in Interphase and Mitotic Cells
HOMER annotatePeaks at shared H3K9ac peak regions



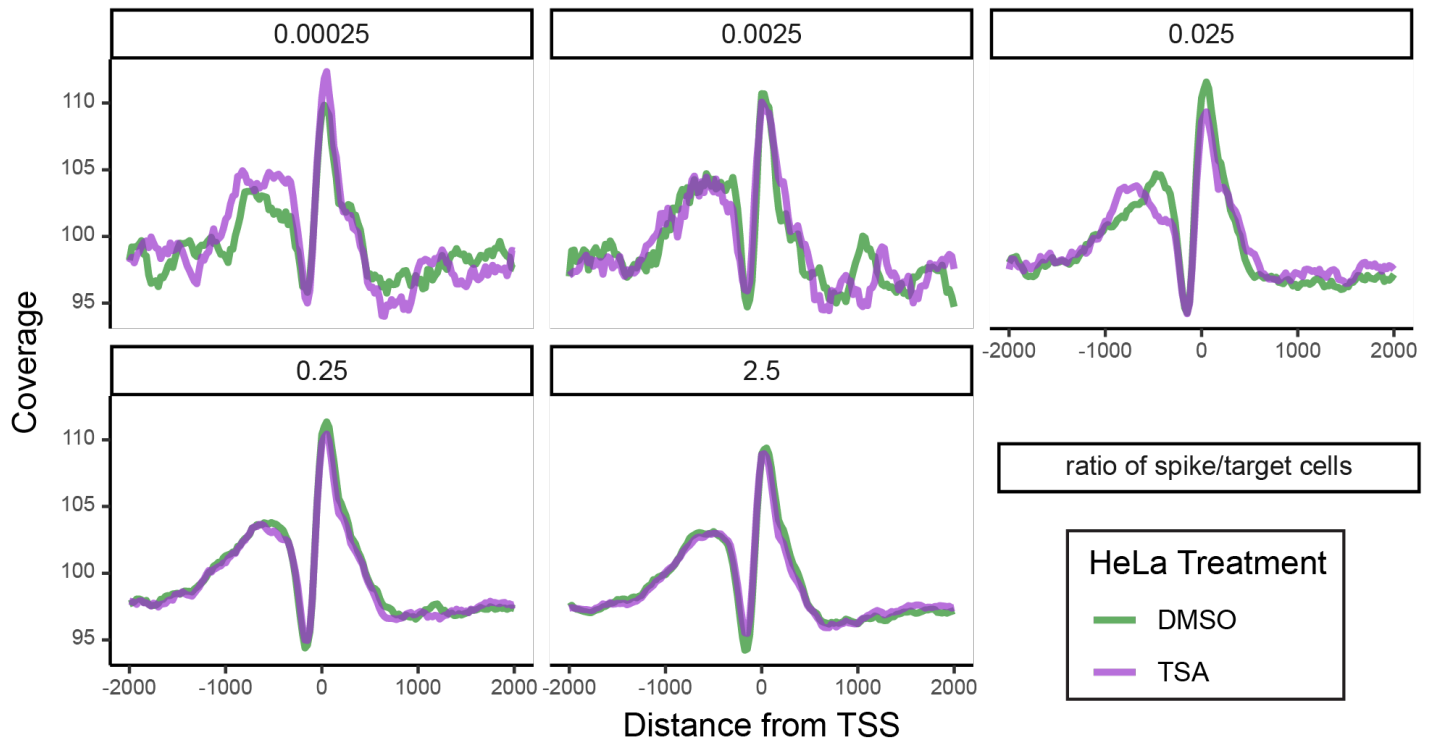
Supplemental Fig. 6. Comparison of Read-depth normalized H3K9ac peaks in mitotic and interphase HeLa-S3 cells. Comparing read-normalized tags in mitotic and interphase cells at the union of all H3K9ac peaks, tag counts averaged between three technical replicates.



Supplemental Fig. 7. Alignment to separate genomes, rather than a concatenated spike-in/target genome, results in excess spike-in aligned reads. Two datasets were analyzed, GSE102882 which contains closely related spike-in (mouse) and target (human) species, and GSE96654 which contains distantly related spike-in (mouse) and target (fly) species. The y axis gives the ratio of separately aligned to concatenated aligned spike-in reads. A lack of excess reads would result in a value of 1, represented by the horizontal line. Importantly, excess alignment is reduced when reads are filtered for primary alignments with MAPQ >10 (in blue). Misassignment and double-counting of reads has a greater influence on results when the spike-in and target genomes are more closely related. When *M. musculus* is used as a spike-in control for *H. sapiens* ChIP-seq or vice versa (found in 15 studies from our survey), separate alignment results in up to 5.4-14.4% excess reads assigned to the spike-in genome compared to alignment using the recommended concatenated genome¹². Notably, this large percent of excess aligned reads appear to be low mapping quality/multimappers; if filtering for primary alignments with MAPQ>10, the proportion of misassigned reads is decreased yet remains in the range of 1.8-4.4% (**Supplemental Table 2**).

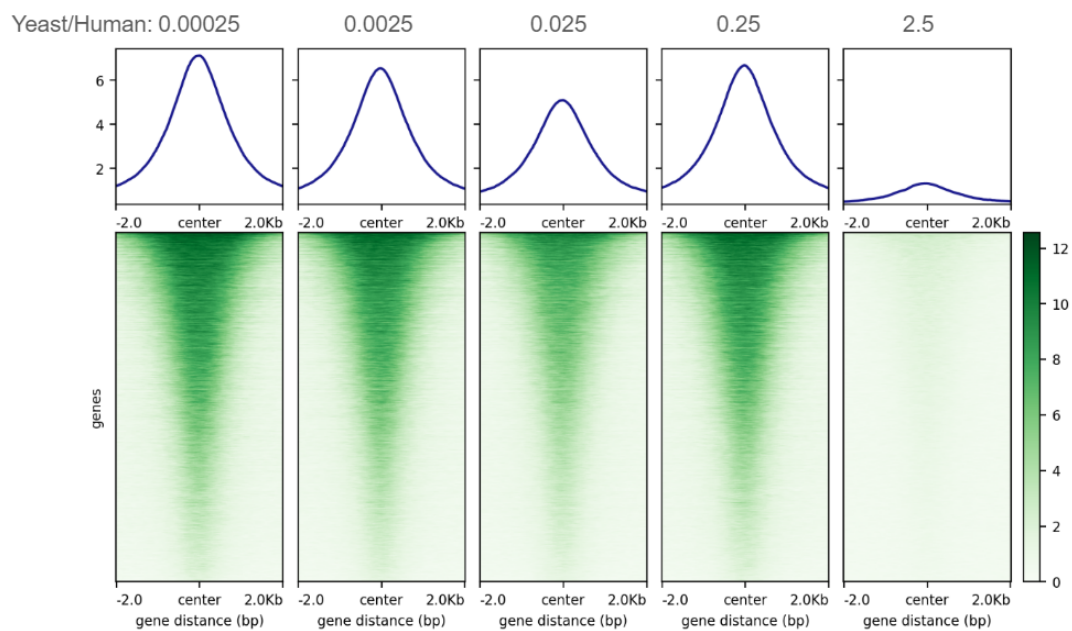


Supplemental Fig. 8. Ratio of yeast-aligned and human-aligned reads in H3K9ac ChIP-seq in Mitotic HeLa-S3 cells. All sequencing statistics and sample information available in **Supplemental Table 4**.



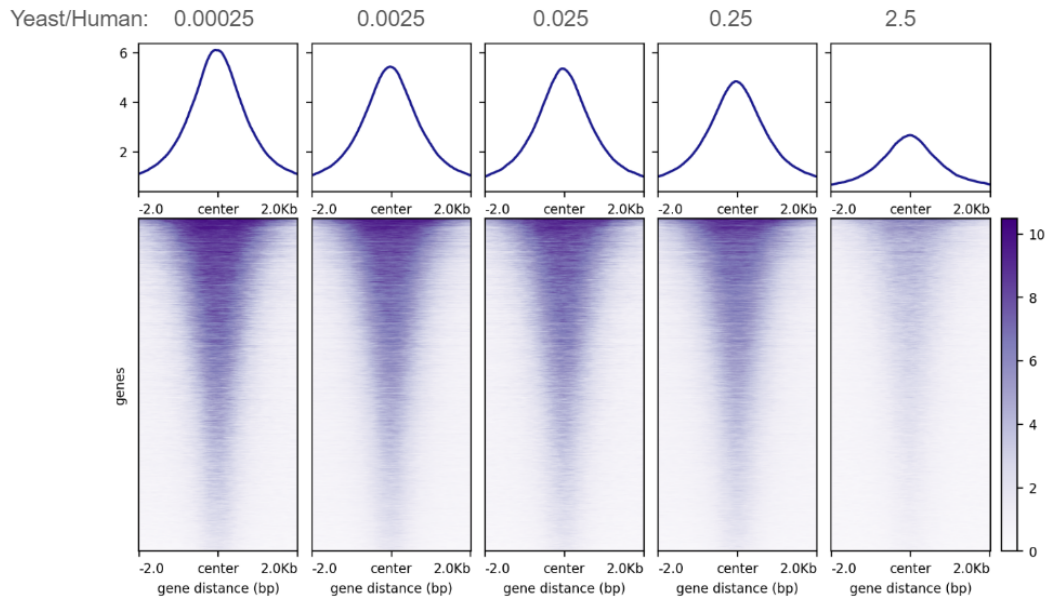
Supplemental Fig. 11. TSS Histograms of spike-in *Saccharomyces cerevisiae* H3K9ac signal as a function of input cell number. The ratio of yeast/human cells was varied by a factor of 10000, from 0.00025-2.5. Histograms generated with HOMER annotatePeaks with parameters: tss sacCer3 -size 4000 -hist 25.

H3K9ac peaks in DMSO-treated Mitotic HeLa-S3 cells

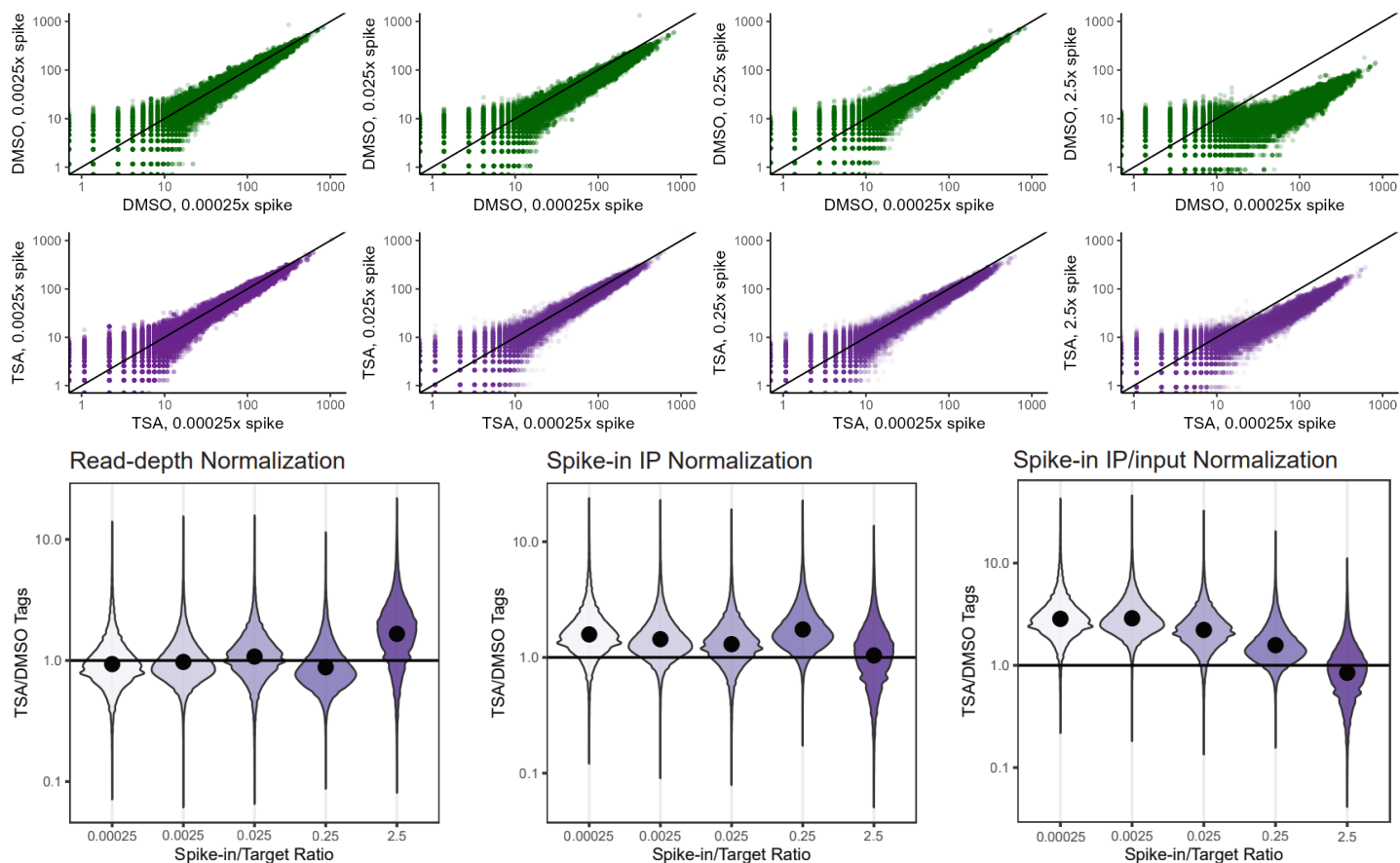


Supplemental Fig. 12. Heatmap of H3K9ac peaks in DMSO treated HeLa-S3 cells. Samples are read-depth normalized. The heatmap was generated using Deeptools computeMatrix with the union set of all peaks called for each sample (see Methods). The ratio of yeast/human cells was varied by a factor of 10000, from 0.00025-2.5.

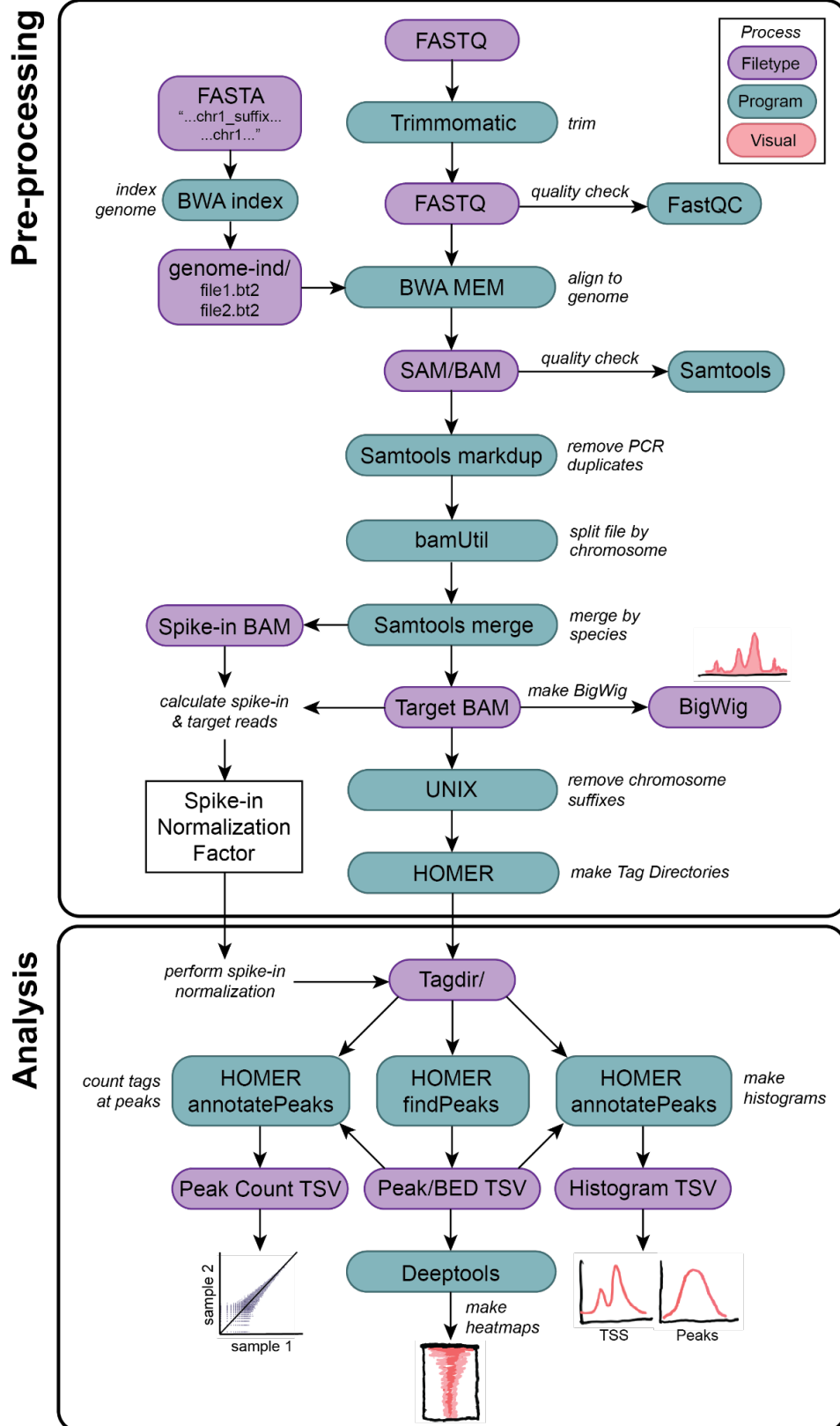
H3K9ac peaks in TSA-treated Mitotic HeLa-S3 cells



Supplemental Fig. 13. Heatmap of H3K9ac peaks in TSA treated HeLa-S3 cells. The heatmap was generated using Deeptools computeMatrix with the union set of all peaks called for each sample (details in Methods). The ratio of yeast/human cells was varied by a factor of 10000, from 0.00025-2.5.



Supplemental Fig. 14. Top: Quantification of H3K9ac at all peaks in DMSO treated mitotic HeLa-S3 cells. Peaks were called for each sample using HOMER findPeaks, all peaks were merged to obtain the union set, then tags were counted with HOMER annotatePeaks. Read normalized tag counts are plotted on a log scale. **Middle:** Quantification of H3K9ac at all peaks in TSA treated mitotic HeLa-S3 cells. **Bottom:** Violin plot of TSA/DMSO H3K9ac signal distribution in mitotic HeLa-S3 cells, comparing normalization strategies (details in Methods).



Supplemental Fig. 15. Schematic representation of data analysis. Code for generating main text figures available on Github: https://github.com/lapatel22/spike_in_correspondence_2024/tree/main.

References

- (1) Orlando, D. A.; Chen, M. W.; Brown, V. E.; Solanki, S.; Choi, Y. J.; Olson, E. R.; Fritz, C. C.; Bradner, J. E.; Guenther, M. G. Quantitative ChIP-Seq Normalization Reveals Global Modulation of the Epigenome. *Cell Reports* **2014**, 9 (3), 1163–1170. <https://doi.org/10.1016/j.celrep.2014.10.018>.
- (2) Javasky, E.; Shamir, I.; Gandhi, S.; Egri, S.; Sandler, O.; Rothbart, S. B.; Kaplan, N.; Jaffe, J. D.; Goren, A.; Simon, I. Study of Mitotic Chromatin Supports a Model of Bookmarking by Histone Modifications and Reveals Nucleosome Deposition Patterns. *Genome Res.* **2018**, 28 (10), 1455–1466. <https://doi.org/10.1101/gr.230300.117>.

Supplemental Table 1. GEO Accession numbers for public datasets and datasets in this study.

Study Title + Reference Number for Supplemental Table 1	GSE	Target	Spike	Status of Inputs	Alignment Strategy
High-sensitive nascent transcript sequencing reveals BRD4-specific control of widespread enhancer and target gene transcription. ¹	GSE214590	human	mouse	appropriate	Aligned to concatenated genome
Induction of senescence-associated secretory phenotype underlies the therapeutic efficacy of PRC2 inhibition in cancer ²	GSE183601	human	fly	appropriate	Aligned separately
Aberrant paracrine signalling for bone remodelling underlies the mutant histone-driven giant cell tumour of bone ³	GSE152921	human	fly	appropriate	Aligned separately
Distinct roles for CKM–Mediator in controlling Polycomb-dependent chromosomal interactions and priming genes for induction ⁴	GSE185927	mouse	human	appropriate	Aligned to concatenated genome
PRC2.1- and PRC2.2-specific accessory proteins drive recruitment of different forms of canonical PRC1 ⁵	GSE199527	mouse	fly	appropriate	Aligned to concatenated genome
Parallel factor ChIP provides essential internal control for quantitative differential ChIP-seq ⁶	GSE102882	human	mouse	appropriate	Aligned to concatenated genome
SIX1 reprograms myogenic transcription factors to maintain the rhabdomyosarcoma undifferentiated state ⁷	GSE173151	human	fly	appropriate	Ambiguous language
A CpG island-encoded mechanism protects genes from premature transcription termination ⁸	GSE199801	mouse	human	appropriate	Aligned to concatenated genome
Whole-genome doubling drives oncogenic loss of chromatin segregation ⁹	GSE222391	human	mouse	appropriate	Aligned to concatenated genome
LEO1 Is Required for Efficient Entry into Quiescence, Control of H3K9 Methylation and Gene Expression in Human Fibroblasts ¹⁰	GSE247829	human	fly	appropriate	Aligned to concatenated genome
Establishment of regions of genomic activity during the Drosophila maternal-to-zygotic transition ¹¹	GSE58935	fly	dp3	appropriate	Aligned to concatenated genome
Transcriptomic and epigenomic analysis of germ-free and colitic mice ¹²	GSE179233	mouse	fly	appropriate	Aligned to concatenated genome
Epigenetic drift of H3K27me3 in aging links glycolysis to healthy longevity in Drosophila ¹³	GSE96654	fly	mouse	appropriate	Aligned separately
Counteracting chromatin effects of a splicing-correcting antisense oligonucleotide improves its therapeutic efficacy in spinal muscular atrophy ¹⁴	GSE167762	human	fly	appropriate	Ambiguous language
Context-dependent perturbations in chromatin folding and the transcriptome by cohesin and related factors ¹⁵	GSE196450	human	mouse	appropriate	Ambiguous language
Quantitative ChIP-Seq Normalization Reveals Global Modulation of the Epigenome ¹⁶	GSE60104	human	fly	appropriate	Aligned to concatenated genome
Single Cell Analyses Reveal a Non-Canonical EZH2 Activity As a Main Driver of Retinoic Acid Resistance in PLZF/Rara Leukemia ¹⁷	GSE181186	mouse	fly	appropriate	Aligned to concatenated genome

The Mediator complex regulates enhancer-promoter interactions ¹⁸	GSE225294	human	fly	appropriate	Ambiguous language
NSD1-deposited H3K36me2 directs de novo methylation in the mouse male germline and counteracts Polycomb-associated silencing ¹⁹	GSE148150	mouse	synth	appropriate	Aligned separately
RNA helicase-dependent gene looping impacts messenger RNA processing ²⁰	GSE183517	human	fly	appropriate	Aligned to concatenated genome
Prediction of histone post-translational modification patterns based on nascent transcription data ²¹	GSE163043	human	diulia	appropriate	Aligned to concatenated genome
PAX5 promotes heterochromatin formation ²²	GSE165035	human	fly	appropriate	Ambiguous language
Histone H3K36me2 and H3K36me3 form a chromatin platform essential for DNMT3A-dependent DNA methylation in mouse oocytes ²³	GSE183969	mouse	synth	appropriate	Ambiguous language
Condensates induced by transcription inhibition localize active chromatin to nucleoli ²⁴	GSE166623	human	fly	appropriate	Aligned separately
An essential role for the Ino80 chromatin remodeling complex in regulation of gene expression during cellular quiescence ²⁵	GSE200378	yeast	fly	appropriate	Aligned separately
Titration of H3K9ac in mitotic and interphase HeLa-S3 cells	GSE273915	human	yeast	appropriate	Aligned to concatenated genome
Titration of H3K9ac in mitotic and interphase HeLa-S3 cells	GSE273915	human	fly	appropriate	Aligned to concatenated genome
SETDB1/NSD-dependent H3K9me3/H3K36me3 dual heterochromatin maintains gene expression profiles by bookmarking poised enhancers ²⁶	GSE171749	mouse	fly	insufficient	Aligned separately
Tnpo3 enables EBF1 function in conditions of antagonistic Notch signaling ²⁷	GSE201527	mouse	human	insufficient	Aligned separately
Context-dependent function of the transcriptional regulator Rap1 in gene silencing and activation in <i>Saccharomyces cerevisiae</i> ²⁸	GSE227761	yeast	yeast	insufficient	Aligned to concatenated genome
Quantifying ChIP-seq data: a spiking method providing an internal reference for sample-to-sample normalization ²⁹	GSE52049	mouse	human	insufficient	Ambiguous language
ChIP-seq of histone modifications and EZH2 in the striatum of four-month-old HttQ111/+ and wildtype mice ³⁰	GSE150747	mouse	fly	insufficient	Aligned separately
The glucocorticoid receptor recruits the COMPASS complex to regulate inflammatory transcription at macrophage enhancers ³¹	GSE138017	mouse	fly	insufficient	Aligned separately
Discovery of a dual WDR5 and Ikaros PROTAC degrader as an anti-cancer therapeutic ³²	GSE175544	human	fly	insufficient	Ambiguous language
β -catenin programs a tissue-specific epigenetic vulnerability in aggressive adrenocortical carcinoma ³³	GSE205258	human	fly (synthetic, Active Motif)	insufficient	Ambiguous language
SF3B1 facilitates HIF1-signaling and promotes malignancy in pancreatic cancer ³⁴	GSE201461	human	fly	insufficient	Aligned to concatenated genome

EZH2 noncanonically binds cMyc and p300 through a cryptic transactivation domain to mediate gene activation and promote oncogenesis ³⁵	GSE150745	human	fly	insufficient	Ambiguous language
Multiomic analysis of Cohesin reveals that ZBTB transcription factors contribute to chromatin interactions ³⁶	GSE184268	mouse	human	insufficient	Aligned separately
Inseparable RNA binding and chromatin modification activities of a nucleosome-interacting surface in EZH2 ³⁷	GSE239445	human	mouse	insufficient	Aligned to concatenated genomes
ChIP-Seq of Wild Type pDC Transcriptomes ³⁸	GSE171868	mouse	fly	lacking inputs	Aligned separately
Proximity-dependent recruitment of Polycomb repressive complexes by the lncRNA Airn ³⁹	GSE217262	mouse	human	lacking inputs	Ambiguous language
LATS1 controls CTCF chromatin occupancy and hormonal response of 3D-grown breast cancer cells ⁴⁰	GSE247777	human	fly	lacking inputs	Ambiguous language
An Alternative Approach to ChIP-Seq Normalization Enables Detection of Genome-Wide Changes in Histone H3 Lysine 27 Trimethylation upon EZH2 Inhibition ⁴¹	GSE63494 and GSE64243	human	fly	lacking inputs	Aligned separately
G4access identifies G-quadruplexes and their associations with open chromatin and imprinting control regions ⁴²	GSE187007	human	fly	lacking inputs	Aligned to concatenated genomes
Active regulatory elements recruit cohesin to establish cell-specific chromatin domains ⁴³	GSE244925	mouse	human	lacking inputs	Aligned to concatenated genomes
RNA Pol II preferentially regulates ribosomal protein expression by trapping disassociated subunits ⁴⁴	GSE225454	mouse	human	lacking inputs	Aligned to concatenated genomes
Targeted protein degradation reveals RNA Pol II heterogeneity and functional diversity ⁴⁵	GSE179967	mouse	fly	lacking inputs	Aligned to concatenated genomes
Cross-species chromatin interactions drive transcriptional rewiring in Epstein-Barr virus-positive gastric adenocarcinoma ⁴⁶	GSE135176	human	fly (synthetic, Active Motif)	lacking inputs	Aligned separately
The impact of SWI/SNF and NuRD inactivation on gene expression is tightly coupled with levels of RNA polymerase II occupancy at promoters ⁴⁷	GSE206497	mouse	fly	lacking inputs	Aligned to concatenated genomes
NELF focuses sites of initiation and maintains promoter architecture ⁴⁸	GSE233930	human	mouse	lacking inputs	Aligned to concatenated genomes
Methylation of histone H3 lysine 4 is required for maintenance of beta cell function in adult mice ⁴⁹	GSE181949	mouse	fly	lacking inputs	Aligned to concatenated genomes
H3K4me3 regulates RNA polymerase II promoter-proximal pause-release ⁵⁰	GSE181714	mouse	fly	lacking inputs	Aligned separately
DOT1L is a barrier to histone acetylation during reprogramming to pluripotency ⁵¹	GSE190391	mouse	human	lacking inputs	Aligned separately

Note: if alignment to target and spike-in genomes were described in separate steps, with no mention of a combined reference genome, then alignment is counted as separate alignment. If the study provides no additional details but cites a previous method, we count alignment strategy by the previous method. If description in the manuscript was not clear regarding the alignment procedure, it was defined as ambiguous.

Methods:

Public Data Analysis

Detailed code is available at the Github repository link in the Additional Data section. The basic procedure for calculating alignment statistics is shown below:

1. All FASTQ files downloaded using sra-toolkit fasterq-dump:

```
$ fasterq-dump --skip-technical --split-3 SRR#####
```

2. FASTQ files aligned with Bowtie2 default parameters to a concatenated reference genome containing spike-in and target genomes. Spike-in chromosomes labeled with suffix of genome version to distinguish from target chromosomes.

```
$ bowtie2 -p 24 -x target_spike-Bowtie2Index/target_spike  
SRR#####.fastq -S SRR#####.dualalign.sam
```

3. Aligned file sorted, then split into species-specific .bam files with bamutil splitChromosome

```
$ samtools sort SRR#####.sam > SRR#####.sorted.bam  
  
$ bam splitChromosome --in SRR#####.sorted.bam -out SRR#####.
```

4. Unmapped, non-primary alignments removed, and reads with MAPQ<10 removed with HOMER makeTagDirectories. Fragment length explicitly set to 150.

```
$ makeTagDirectories SRR#####-tagdir/ -genome genome -fragLength  
150 SRR#####.genome.bam
```

NOTE: The number of aligned reads for paired end Read 1 may not equal Read 2, therefore “total tags” may be reported in intervals of 0.5.

5. Total read counts tallied in **Supplementary Table 1**.

Genome versions:

H. sapiens: hg38

D. melanogaster: dm6

D. pseudoobscura: dp3

M. musculus: mm10

D. iulia: GCA_019049465.1

Synthetic nucleosome sequences from Epicypher: Kmet_stat panel

Experimental Methods

Cell Culture

HeLa-S3 Cell Media and Culture: HeLa-S3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1x penicillin-streptomycin, L-glutamine, and 110mg/L sodium pyruvate. Cells were incubated at 37°C and 5% CO₂.

S2 Cell Culture: Drosophila S2 cells were grown in Schneiders Drosophila Medium at 28°C in the dark.

Yeast Cell Culture: From frozen stocks, starter cultures of strain LPY6494 were inoculated in 3mL of Gibco liquid YPD media (Cat #A1374501), grown to log phase at 30°C at 220rpm, then expanded to 50mL cultures at an OD of 0.08 and grown for 16 hours at 30°C and 220rpm. OD was estimated with Nanodrop.

HeLa-S3 Mitotic Synchronization

Cells were synchronized in G1/S by adding 2 mM thymidine (Sigma T1895-1G) for 16 hr, washed with PBS, released for 3 hr in fresh medium, and pro-metaphase arrested with 10uM S-trityl-L-cysteine (STC) (Sigma 164739) for 12 hr then harvested. For simplicity, we term these cells as "mitotic", even though according to our estimates approximately 85-90% of cells are arrested in prometaphase. When specified, cells were treated with DMSO or 150nM TSA (Selleck Chemicals S1045) 9 hours before harvesting.

ChIP-seq

ChIP-seq was performed as previously described⁵². Briefly, HeLa-S3 cells were double crosslinked on plates with 2 mM DSG for 30 minutes (Fisher Scientific NC1736108) and 1% formaldehyde (Pierce ThermoFisher PI28906) for 10 minutes. Crosslinking was quenched with 1/20th volume of 2.625 M glycine (EMD Millipore 357002) and 0.5% BSA (NEB B9000S), then cells were washed 3 times with 0.5% BSA/PBS + Protease inhibitor (PIC, Roche 11697498001), spinning down at 1200g for 5 minutes at 4C. Washed pellets were snap frozen then stored in -80C. Cells were lysed with LB3 lysis buffer + PIC, then chromatin was sheared on the PIXUL sonicator for 108 minutes (Active Motif 53130). S2 cells were crosslinked in 1% formaldehyde for 10 minutes at room temperature, then quenched with glycine and 0.5% BSA, lysed in LB3, and sonicated on the PIXUL for 60 minutes. Yeast cultures in log phase were crosslinked with 0.86% formaldehyde for 30 minutes. The yeast cell wall was digested with Zymolase at 37°C for 60 minutes, cells were washed 3x in PBS + PI, then resuspended in NP-S buffer + PI. Yeast were

sonicated on the PIXUL for 120 minutes. All PIXUL sonication was done with settings Pulse (N) = 50, PRF (kHz) = 1.00, Burst Rate (Hz) = 20, only Process Time was varied.

Sheared chromatin from each species were combined and then inputs were taken before immunoprecipitation for each condition to assess the relative ratios of chromatin. For the experiment detailed in **Fig. 1b**, where H3K9ac titrated in mitotic and interphase HeLaS3 cells, chromatin from approximately 600k HeLaS3 cells was combined with chromatin from 500k *S. cerevisiae* and 100k *D. melanogaster* S2 cells in each IP. For the experiment detailed in **Fig. 4**, where we titrated *S. cerevisiae* spike-in amounts, chromatin from *S. cerevisiae* was added in ratios ranging from 0.00025-2.5x relative to HeLaS3 cells.

Notes on minimizing variability between spike-in/target ratios: Since there are multiple opportunities between cell counting and combining of material from each species that may introduce variability, it is difficult to maintain constant spike-in/target chromatin ratios. To overcome this challenge, rather than relying on cell count estimates, we measure sheared DNA quantities as means to estimate chromatin amounts prior to combining material from each species.

Thus, following shearing by PIXUL, a subset of chromatin was de-crosslinked, the RNA was removed with RNase A (Roche-SIGMA 11119915001) and protein was removed with Proteinase K (Invitrogen 25530049). The resulting DNA is quantified with Qubit HS DNA kit (Invitrogen Q32854). Then the samples can be calibrated such that equal amounts of spike-in and target chromatin are added for every condition. Except for the experiment where yeast chromatin was deliberately varied, the estimated number of yeast cells added per IP is no more than 1x the number of human cells. As the *S. cerevisiae* genome is gene dense and the epitope of interest is a histone acetylation mark, *S. cerevisiae* makes up a significant proportion of reads in the IP (**Supplemental Table 3**). In a sample with minimal target IP signal, e.g. 100% STC-treated (mitotic) cells, *S. cerevisiae* accounts for ~0.8% of the input reads and ~30% of IP reads from the same chromatin pool (**Supplemental Table 3, Supplemental Fig. 2**).

To each IP, 10uL of Protein A beads (Thermo Fisher Scientific 10002D) and 4uL of H3K9ac (CST 9649) were added, 1% Triton X-100 and 1x PIC were added. Samples were incubated overnight on rotator at 4C. IP Washes were performed as follows: 3x with 180uL of WBI, 3x with 180uL of WBIII, and 2x with 185uL TET, then eluted in 25uL of cold TT Buffer (buffer components below). Inputs were incubated with Speedbeads (Cytiva 65152105050250), in 12% PEG (Millipore Sigma P5413) and 1M NaCl for 10 minutes at room temperature, then washed twice with 80% EtOH, and eluted in 25uL cold TT Buffer. From inputs and IPs, libraries were prepared with the NEB Ultra II Library Prep Kit (NEB E7645L). Barcoded adapters were from BioO NEXTflex ChIP-seq

Adapters (NOVA-514104 & NOVA-514151). Libraries were de-crosslinked at 55C 1hr, then 65C overnight, RNA removed with RNase A (Roche-SIGMA 11119915001) and protein removed with Proteinase K (Invitrogen 25530049), and cleaned with Speedbeads, 8.6% PEG and 0.8M NaCl. Next, libraries were PCR amplified with 1uM Solexa 1GA and 1GB primers and NEB Ultra II Q5 Mastermix (PCR protocol: 98C for 30", then 12 cycles of 98C for 10", 60C for 15", 72C for 30", then hold at 72C for 2'). A final cleanup was done with Speedbeads, 8.5% PEG and 1M NaCl, washed twice with 80% EtOH, then the final libraries were eluted in 20uL TT. Library size was estimated on a 2% agarose gel and concentration quantified with Qubit HS dsDNA kit (Invitrogen Q32854). Libraries were pooled and sequenced on Illumina NextSeq 550.

Buffer components:

NP-S Buffer: 10mM Tris-HCl pH 7.4, 0.5mM EDTA, 50mM NaCl, 5mM MgCl₂, 1mM CaCl₂, 0.075% NP-40, 1mM 2-mercaptoethanol

LB3: 10mM Tris-HCl pH 7.5, 100mM NaCl, 1mM EDTA, 0.1% deoxycholate, 0.5% Sarkosyl

WBI: 20mM Tris-HCl pH 7.5, 150mM NaCl, 1% Triton X-100, 2mM EDTA, 1% SDS

WBIII: 10mM Tris-HCL pH 7.5, 250mM LiCl, 1% Triton X-100, 1mM EDTA, 0.7% deoxycholate

TET: 10mM Tris-HCl pH 8.0, 1mM EDTA, 0.2% Tween 20

TT Buffer: 10 mM Tris-HCl pH 8.0, 0.05% Tween 20

Bioinformatic Analysis

An overview of data processing and analysis steps is shown in **Supplemental Fig. 15**, and details that vary between datasets are described below.

Data Processing and Initial Analysis

Samples were demultiplexed with Illumina's bcl2fastq program, adapters were removed with Trimmomatic, and FASTQC was run for sequencing quality control. Reads were aligned with BWA MEM to a concatenated genome containing hg38 + spike-in species (dm6, sacCer3, or both). The aligned sam file was sorted and split by chromosome using bamUtil splitChromosome. Spike-in chromosomes were pre-labeled with a suffix (_sac3 or _dm6), samtools merge was used to recombine alignments for each species. Duplicate reads were removed with samtools markdup. For easier downstream processing, HOMER tag directories were made; by default read counts for each species were filtered for primary alignments with MAPQ > 10. Signal was quantified at peaks with HOMER annotatePeaks. Peak finding for each individual sample was done with HOMER findPeaks, with -style histone, -size 1000 -minDist 2500 with the corresponding inputs

as is recommended for variable length peaks: <http://homer.ucsd.edu/homer/ngs/peaks.html>; FRIP score for each sample was estimated in output file of peak finding.

For the yeast spike-in titration dataset, scatterplots of peak counts were generated to compare spike-in titration conditions in **Supplemental Fig. 14**. Sample IPs were combined into one tag directory, and all inputs combined similarly, then HOMER findPeaks was done with the recommended variable length peak parameters on this “mega-sample” using the “mega-input” as the input control. The increased read-depth of both sample and input files will increase the tag threshold for peak finding, resulting in more significant peaks. Read-depth normalized signal at each peak was quantified with HOMER annotatePeaks using mega-sample peaks to determine quantified regions, with additional parameters -size 1000. For each treatment condition (TSA or DMSO), signal in each increasing yeast ratio (0.0025-2.5x) were plotted against the sample with the minimum yeast ratio (0.00025x).

Visualization

Bigwig files were created with HOMER makeBigWig and viewed on the UCSC Genome Browser. Heatmaps were generated using Deeptools computeMatrix and plotHeatmap with the union set of all peaks called for each experiment, with additional parameters: reference-point --referencePoint center -b 2000 -a 2000. Scatterplots were generated from HOMER annotatePeaks quantification of signal at peak or tss regions as specified in figure captions. Plots generated with R, ggPlot2, and cowplot.

Normalization

ChIP-Rx titration of H3K79me2: ChIP-Rx dataset from Orlando et al.¹ was normalized according to the previously published method, with additional steps outlined below. The experiment was performed in human Jurkat cells, with *Drosophila* S2 cells as a spike-in. Therefore, all previously described alignment steps were followed with *D. melanogaster* and *H. sapiens* alignments, with *Drosophila* chromosomes labeled with the “_dm6” suffix. The initial ChIP-Rx method determines the normalization factor as $\alpha = \frac{1}{N_d}$, where N_d = (number of reads aligning to the *Drosophila* genome/1000000)¹. We additionally applied an adjustment factor accounting for the variability in spike-in/target chromatin present in the input samples. The ratio of spike-in/target input reads is defined by the equation $R_{input} = \frac{spike_{input}}{target_{input}}$. R_{input} was scaled as in **Fig. 3** by $R_{input,scaled} =$

$\frac{R_{input,i}}{\min(R_{input})}$ so that the sample with the lowest R_{input} is set to 1. The final normalized signal is

therefore calculated as $z_i = x_i * \left(\frac{1}{N_d}\right) * R_{input,scaled}$

As H3K79me2 is predominantly located on promoters, we quantified signal at TSS using similar parameters as Orlando et al., measuring 5kb upstream and downstream of the TSS with HOMER annotatePeaks.pl tss hg38 -size 10000 -hist 25. TSS histograms were made first with traditional read-normalization (the default behavior of HOMER annotatePeaks). Spike-in normalization was done by multiplying read-normalized histogram signal by the spike-in normalization factor “IP_input_factor” for each sample.

Titration of H3K9ac: The dataset containing a titration of mitotic and interphase HeLaS3 cells includes spike-in chromatin from two separate species, *D. melanogaster* and *S. cerevisiae*. Alignment was done against the concatenated genome of hg38 + dm6 + sacCer3. After splitting alignment files into each species, removing PCR duplicates, and filtering reads by making HOMER tag directories, reads aligning to each species were quantified as shown in **Supplemental Table 3**. Normalization factors for each spike-in species were determined as follows: norm_factor = (spike-in IP reads/target IP reads) * (target input reads/spike-in input reads). Normalization factors for each spike-in species, named “fly norm factor”, and “yeast norm factor” are listed in **Supplemental Table 3**. H3K9ac is present on both promoters and enhancers, so we sought to quantify signal at a set of H3K9ac peaks across all samples in the experiment. Sample IPs were combined into one tag directory, and all inputs combined similarly, then HOMER findPeaks was done with the recommended variable length peak parameters on this “mega-sample” using the “mega-input” as the input control. The increased read-depth of both sample and input files will increase the tag threshold for peak finding, resulting in more significant peaks. H3K9ac signal was quantified at these peaks with HOMER annotatePeaks, using the mega-sample peak file as first argument to define quantified regions, then additional parameters hg38 -size 4000 -hist 25. Spike-in normalization was done by dividing read-normalized histogram signal by the spike-in normalization factor “fly norm factor” or “yeast norm factor” for each sample. $z_i =$

$$x_i * \left(\frac{target\ IP}{spike-in\ IP}\right) * \left(\frac{spike-in\ input}{target\ input}\right)$$

Titration of *S. cerevisiae* spike-in: The spike-in titration dataset, comparing TSA and DMSO treated mitotic HeLa-S3 cells contained *S. cerevisiae* chromatin, added in ratios ranging from 0.00025-2.5x that of the target chromatin. Alignment was done against the concatenated genome of hg38 + sacCer3. After splitting alignment files into each species, removing PCR duplicates, and filtering reads by making HOMER tag directories, reads aligning to each species were quantified

as shown in **Supplemental Table 4**. Spike-in normalization was performed according to two commonly used strategies: 1) normalizing to spike-in IP alone, and 2) normalizing to both spike-in IP and input chromatin.

- 1) The normalization factor was calculated as the ratio of yeast/human reads multiplied by a scaling factor of 200 to account for relative genome size and is reported in **Supplemental Table 4** as “yeast/human reads*200”. When normalizing to spike-in IP alone, data were normalized by dividing by the normalization factor, “yeast/human reads*200” column for each IP sample. Signal normalized to spike-in IP is calculated as $z_i = x_i * \left(\frac{\text{target IP}}{\text{spike-in IP}} \right)$.
- 2) When normalizing to spike-in IP and input, the normalization factor “yeast/human reads*200” for each IP was divided by the corresponding input value in the same column. The resulting normalization factor accounting for IP and input is reported in **Supplemental Table 4** as the “spike_IP_input_norm”. Spike-in normalization was done by multiplying read-normalized histogram signal by the spike-in normalization factor “IP_input_factor” for each sample. Spike-in normalization was done by dividing read-normalized histogram signal by the spike-in normalization factor “IP_input_factor” for each sample. The final normalized signal z_i is calculated as $z_i = x_i * \left(\frac{\text{target IP}}{\text{spike-in IP}} \right) * \left(\frac{\text{spike-in input}}{\text{target input}} \right)$

Quantification of Normalized Signal

ChIP-Rx titration of H3K79me2 & Titration of H3K9ac: Total signal was estimated by calculating area-under-the-curve for both read-depth normalized and spike-in normalized histograms (see Github). Total signal for each sample was then min-max normalized by the equation $z_i = \left(\frac{x_i - \min(x)}{\max(x) - \min(x)} \right)$. Here, $\min(x)$ is the average minimum signal (H3K79me2 titration: 100% treated cells, H3K9ac titration: 100% mitotic cells), $\max(x)$ is the average maximum signal (H3K79me2 titration: 0% treated cells, H3K9ac titration: 0% mitotic cells). x_i is the signal for each sample and z_i is the minmax normalized signal for each sample. The resulting data is scaled such that the average minimum signal is set at 0, and the average maximum signal is set at 1. This allows more intuitive comparison across datasets and normalization methods. The accuracy of each normalization method is quantified by calculating the R^2 value of data against the line of expected signal (diagonal line shown in plots of **Fig. 1**). For the minmax normalized data, the line of expected signal is simply the diagonal line connecting maximum ($y=1$) and minimum ($y=0$) signal.

R^2 is calculated by the equation $R^2 = 1 - \left(\frac{SS_{res}}{SS_{total}} \right)$ where $SS_{res} = \sum((z_i - expected_i)^2)$ and $SS_{total} = \sum((z_i - mean(z_i))^2)$. R^2 values are reported in **Fig. 1** as inserts in plots.

Titration of *S. cerevisiae* spike-in: While there is existing evidence of the effect of TSA on increasing histone acetylation in mitotic HeLaS3 cells, this dataset contains no known maximum/minimum values with which to construct a standard curve. Therefore, H3K9ac signal was quantified at every peak with HOMER annotatePeaks -size 1000, and the distribution of signal across peaks were shown as violin plots, comparing read-normalized (**Fig. 4a**), spike-in IP normalized (**Fig. 4b**), and spike-in IP and input normalized (**Fig. 4c**) peak counts. Additionally, the ratio of TSA/DMSO counts in each spike-in titration condition were plotted for each normalization method (**Supplemental Fig. 14**).

Data and Code Availability

Public data used to generate **Fig. 1a** are at GSE60104. UCSC Genome Browser sessions available ([H3K9ac titration data](#), [spike-in titration human data](#), [spike-in titration yeast data](#)). Code is on Github at https://github.com/lapatel22/spike_in_correspondence_2024/tree/main.

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