nature research

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Last updated by author(s): Apr 22, 2019

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	LightCycler® 480 System Performance Data Software BMG Labtech Control Software MARS Data Analysis Software
Data analysis	FastQC (v. 0.10.1), BOWTIE2 (v. 2.3.2), BWA (v. 0.7.5), SAMtools (v. 1.6), BEDtools (2.25.0), Integrative Genomics Viewer (IGV v. 2.3), UCSC genome browser, MACS (v. 2.1.2), TopHat (v. 2.0.9), Cufflinks (v. 2.2.1), Cuffdiff, R (v. 3.2.1), Prism (v. 8), Excel (v. 16.18), Deeptools (v. 3.0.2)

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

Datasets are generated during the current study are deposited in the NCBI Gene Expression Omnibus using series accession number GSE114551 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114551). Subseries are GSE 114567 (ATAC-seq), GSE114548 (ChIP-seq), GSE114549 (RNA-seq).

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	RNA-seq studies were performed at least in duplicate for use with the DESeq2 pipeline. ATAC-seq studies were performed in duplicate .The majority of the ChiP-Seq experiments were performed at least in duplicate, and for HIRA and H3.3, with two independent clonal KO mESCs. Biochemical figures were generated with a variety of replicates as reported in the figure legends of our manuscript.
Data exclusions	No data were excluded from the analysis.
Replication	Critical ChIP-seq and RNA-seq data sets were obtained in duplicate. Many ChIP-seq data sets were validated with independent ChIP-qPCR assays and/or experiments with independent KO mESCs. All western blots and related experiments were performed in duplicate or more. Results obtained were consistent.
Randomization	Random allocation is not relevant to our study, since we are performing molecular and genomic analysis of known KO mESCs.
Blinding	Blinding was used to score differentiation state in our mESC colony formation assay. For the other experiments blinding was not relevant to our study; our techniques were all molecular biological techniques where the experimenter designs and executes the experimental conditions so blinding is not possible.

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	H3 general (ab1791, Abcam, Lot # GR177884-2), H3.3 (09-838, Millipore, Lot # 2578126), H3K4me1 (ab8895, Abcam, Lot # GR193882-2), H3K27ac (39133, Active Motif. Lot # 31814008), H3K27ac (39685, Active Motif, Lot # 14517014), H3K64ac (ABE1057, Millipore, Lot # 2826017), H3K122ac (ab33309, Abcam, Lot # GR284790-3), H3K18ac (ab1191, Abcam, Lot # GR3211480-1), H3.1/2 (ABE154, Millipore), p300 (sc-584, Santa Cruz, Lot # F3016), Gapdh (2118, Cell Signaling, Lot # 10), Lamin A (ab26300, Abcam, Lot # GR155781-1), b-Tubulin (T5201, Sigma, Lot # 088K4832), H3S10ph (39253, Active Motif, Lot # 8308001), H3S28ph (07-145, Millipore, Lot # 27707), H3.3S31ph (61671/61672, Active Motif, Lot # 4415001), HIRA (WC15 WC119 hybridoma), DAXX (07-471, Millipore, Lot # 2841952), ATRX (ab97508, Abcam, Lot # GR267227-8), Oct4 (sc-5279, Santa Cruz, Lot # C3109), anti-mouse IgG-HRP (NA93V, GE, Lot # 9773218), anti-rabbit IgG-HRP (170-6515, Biorad, Lot # 350003248), Spike-In antibody (61686, Active Motif, Lot# 00419007)
Validation	Many histone modification antibodies, including several used in our study, have been validated using Brian Strahl's histone tail peptide array (http://histoneantibodies.com). We validated the H3.3, HIRA, ATRX, and DAXX antibodies using our KO mESCs. We validated acetyl and phospho antibodies using peptide dot blots.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	H3.3 WT and KO mESCs - Banaszynski et al., Cell 2013; WT and ATRX KO and DAXX KO mESCs- Sadic et al., EMBO Rep 2015;

Cell line source(s)	WT and HIRA KO mESCs- Roberts et al., Mol Cell Biol 2002; WT and HIRA KO mESCs - this study 293T were purchased from ATCC MEFi ("feeder") were derived from mice and subjected to radiation to mitotically inactivate the cells.
Authentication	KO cell lines were validated by sequencing of genomic DNA and by immunoblot of the protein product.
Mycoplasma contamination	All cell lines tested were negative for mycoplasma. Cells were tested monthly.
Commonly misidentified lines (See ICLAC register)	None

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.

ChIP-seq

Data deposition

☒ Confirm that both raw and final processed data have been deposited in a public database such as GEO.

☒ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links *May remain private before publication.*	To review GEO accession GSE114551: Go to https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114551 Enter token qxenasoitnqpxyr into the box
Files in database submission	RNA-seq ESC_H33WT_RNA-seq_rep1 ESC_H33WT_RNA-seq_rep2 ESC_H33KO_RNA-seq_rep1 ESC_H33KO_RNA-seq_rep2 EB_H33WT_RNA-seq_rep1 EB_H33WT_RNA-seq_rep2 EB_H33KO_RNA-seq_rep1 EB_H33KO_RNA-seq_rep2 ESC_HIRAWT1_RNA-seq_rep1 ESC_HIRAWT1_RNA-seq_rep2 ESC_HIRAKO1_RNA-seq_rep1 ESC_HIRAKO1_RNA-seq_rep2 ESC_HIRAWT2_RNA-seq_rep1 ESC_HIRAWT2_RNA-seq_rep2 ESC_HIRAKO2_RNA-seq_rep1 ESC_HIRAKO2_RNA-seq_rep2 ATAC-seq ESC_H33WT_ATAC ESC_H33KO_ATAC ESC_HIRAWT_ATAC ESC_HIRAKO1_ATAC EB_H33WT_ATAC EB_H33KO_ATAC ChIP-seq ESC_H33WT_H3K27ac_pAb ESC_H33KO_H3K27ac_pAb ESC_H33WT_Input ESC_H33KO_Input ESC_H33WT_H3K27ac_mAb ESC_H33KO_H3K27ac_mAb ESC_H33WT_H3K4me1_rep1 ESC_H33KO_H3K4me1_rep1 ESC_H33WT_H3K4me1_rep2 ESC_H33KO_H3K4me1_rep2 ESC_H33WT_H3K64ac ESC_H33KO_H3K64ac

ESC_H33WT_H3K122ac
ESC_H33KO_H3K122ac
ESC_HIRAWT_H3K27ac
ESC_HIRAKO1_H3K27ac
ESC_HIRAWT_Input
ESC_HIRAKO1_Input
ESC_ATRXDAXXWT_H3K27ac
ESC_ATRXKO_H3K27ac
ESC_DAXXKO_H3K27ac
ESC_ATRXDAXXWT_Input
ESC_ATRXKO_Input
ESC_DAXXKO_Input
ESC_HIRAWT2_H3K27ac
ESC_HIRAKO2_H3K27ac
ESC_HIRAWT2_Input
ESC_HIRAKO2_Input
EB_H33WT_H3K27ac_rep1
EB_H33WT_H3K27ac_rep2
EB_H33KO_H3K27ac_rep1
EB_H33KO_H3K27ac_rep2
EB_H33WT_Input
EB_H33KO_Input
EB_H33WT_H3K27ac
EB_H33KO_H3K27ac
EB_H33KO_H32_H3K27ac
EB_H33KO_H33_H3K27ac
EB_H33KO_S31A_H3K27ac
EB_H33KO_S31E_H3K27ac
ESC_H33WT_p300
ESC_H33KO_p300
ESC_H33WT_Input_p300
ESC_H33KO_Input_p300
ESC_ATRXDAXXWT_H33
ESC_ATRXKO_H33
ESC_DAXXKO_H33
ESC_ATRXDAXXWT_Input_H33
ESC_ATRXKO_Input_H33
ESC_DAXXKO_Input_H33
ESC_H33WT_H3K18ac
ESC_H33KO_H3K18ac
ESC_H33WT_H33S31P
ESC_H33WT_H33S31P_DMSO
ESC_H33WT_H33S31P_Chk1i
ESC_H33WT_H3K27ac_DMSO
ESC_H33WT_H3K27ac_Chk1i
ESC_H33WT_H3K27ac_AuroraBi

Genome browser session
(e.g. UCSC)

http://genome.ucsc.edu/cgi-bin/hgTracks?db=mm10&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr17%3A35506032%2D35510777&hgsid=668907157_UsThPYEQZXkjoaCD2CF2oED4caLw

Methodology

Replicates

The following experiments were performed in replicate and are in high agreement:
WT/H3.3 KO mESC H3K4me1 ChIP-seq
WT/H3.3 KO mESC H3K27ac ChIP-seq
WT/H3.3 KO EB H3K27ac ChIP-seq
In another sense, the H3.3 KO and HIRA KO samples serve as biological replicate demonstrating the pathway responsible for our observations.

Sequencing depth

Sample / Total Reads / Unique Reads / Read length / PE or SE
RNA-seq
ESC_H33WT_RNA-seq_rep1 39,152,592 36,983,355 75 bp PE
ESC_H33WT_RNA-seq_rep2 37,095,065 35,092,925 75 bp PE
ESC_H33KO_RNA-seq_rep1 38,675,988 36,453,961 75 bp PE
ESC_H33KO_RNA-seq_rep2 38,259,977 36,042,510 75 bp PE
EB_H33WT_RNA-seq_rep1 32,802,966 30,875,934 75 bp PE
EB_H33WT_RNA-seq_rep2 37,095,745 35,103,958 75 bp PE
EB_H33KO_RNA-seq_rep1 37,394,096 35,290,777 75 bp PE
EB_H33KO_RNA-seq_rep2 37,281,876 35,113,024 75 bp PE
ESC_HIRAWT1_RNA-seq_rep1 59,081,868 55,403,722 75 bp PE
ESC_HIRAWT1_RNA-seq_rep2 44,774,879 42,751,379 75 bp PE
ESC_HIRAKO1_RNA-seq_rep1 40,676,497 38,829,512 75 bp PE
ESC_HIRAKO1_RNA-seq_rep2 49,642,924 47,352,169 75 bp PE
ESC_HIRAWT2_RNA-seq_rep1 40,836,408 39,056,154 75 bp PE

ESC_HIRAWT2_RNA-seq_rep2 50,653,012 48,185,546 75 bp PE
ESC_HIRAKO2_RNA-seq_rep1 49,334,622 47,082,156 75 bp PE
ESC_HIRAKO2_RNA-seq_rep2 50,993,462 48,762,189 75 bp PE
ATAC-seq
ESC_H33WT_ATAC 56,067,263 36,567,115 75 bp PE
ESC_H33KO_ATAC 59,541,728 38,843,171 75 bp PE
ESC_HIRAWT_ATAC 4,452,232 4,104,854 75 bp PE
ESC_HIRAKO1_ATAC 4,750,640 4,424,468 75 bp PE
EB_H33WT_ATAC 23,125,315 19,554,683 75 bp PE
EB_H33KO_ATAC 37,259,589 30,188,597 75 bp PE
ChIP-seq
ESC_H33WT_H3K27ac_pAb 27,721,672 20,853,968 75 bp PE
ESC_H33KO_H3K27ac_pAb 32,576,988 21,541,644 75 bp PE
ESC_H33WT_Input 46,535,961 34,402,586 75 bp PE
ESC_H33KO_Input 43,266,270 32,198,622 75 bp PE
ESC_H33WT_H3K27ac_mAb 56,583,927 38,913,962 75 bp PE
ESC_H33KO_H3K27ac_mAb 61,580,247 38,623,146 75 bp PE
ESC_H33WT_H3K4me1_rep1 26,787,090 19,016,034 75 bp PE
ESC_H33KO_H3K4me1_rep1 22,689,168 16,032,683 75 bp PE
ESC_H33WT_H3K4me1_rep2 26,696,169 19,523,708 75 bp PE
ESC_H33KO_H3K4me1_rep2 32,598,169 21,268,680 75 bp PE
ESC_H33WT_H3K64ac 16,883,148 7,520,020 75 bp PE
ESC_H33KO_H3K64ac 21,452,815 8,335,361 75 bp PE
ESC_H33WT_H3K122ac 21,785,220 14,507,357 75 bp PE
ESC_H33KO_H3K122ac 22,447,652 13,607,496 75 bp PE
ESC_HIRAWT_H3K27ac 15,668,253 10,798,859 75 bp PE
ESC_HIRAKO1_H3K27ac 24,130,435 15,767,613 75 bp PE
ESC_HIRAWT_Input 44,523,090 32,342,782 75 bp PE
ESC_HIRAKO1_Input 40,815,945 27,352,420 75 bp PE
ESC_ATRXDAXXWT_H3K27ac 21,868,016 16,499,473 75 bp PE
ESC_ATRXKO_H3K27ac 17,456,727 12,306,771 75 bp PE
ESC_DAXXKO_H3K27ac 14,235,183 10,706,317 75 bp PE
ESC_ATRXDAXXWT_Input 37,190,762 26,758,554 75 bp PE
ESC_ATRXKO_Input 33,430,249 22,784,831 75 bp PE
ESC_DAXXKO_Input 35,875,187 25,751,655 75 bp PE
ESC_HIRAWT2_H3K27ac 30,247,397 23,814,769 75 bp PE
ESC_HIRAKO2_H3K27ac 35,038,719 26,515,021 75 bp PE
ESC_HIRAWT2_Input 13,895,594 9,913,264 75 bp PE
ESC_HIRAKO2_Input 16,106,172 11,648,982 75 bp PE
EB_H33WT_H3K27ac_rep1 57,057,343 44,786,024 75 bp PE
EB_H33WT_H3K27ac_rep2 31,423,951 23,605,253 75 bp PE
EB_H33KO_H3K27ac_rep1 64,745,600 49,468,119 75 bp PE
EB_H33KO_H3K27ac_rep2 39,399,995 29,964,559 75 bp PE
EB_H33WT_Input 72,516,125 53,345,957 75 bp PE
EB_H33KO_Input 77,968,453 56,660,871 75 bp PE
EB_H33WT_H3K27ac 36,033,793 27,366,742 75 bp PE
EB_H33KO_H3K27ac 38,303,796 29,492,642 75 bp PE
EB_H33KO_H32_H3K27ac 38,630,142 29,551,504 75 bp PE
EB_H33KO_H33_H3K27ac 38,712,513 29,560,333 75 bp PE
EB_H33KO_S31A_H3K27ac 39,847,067 31,025,523 75 bp PE
EB_H33KO_S31E_H3K27ac 39,910,159 32,087,319 75 bp PE
ESC_H33WT_p300 28,334,526 33,454,023 75 bp PE
ESC_H33KO_p300 32,251,079 22,409,907 75 bp PE
ESC_H33WT_Input_p300 24,163,122 17,880,747 75 bp PE
ESC_H33KO_Input_p300 29,377,010 21,338,366 75 bp PE
ESC_ATRXDAXXWT_H33 35,692,205 25,608,331 75 bp PE
ESC_ATRXKO_H33 34,691,122 24,301,991 75 bp PE
ESC_DAXXKO_H33 32,322,499 23,964,883 75 bp PE
ESC_ATRXDAXXWT_Input_H33 23,618,265 17,934,071 75 bp PE
ESC_ATRXKO_Input_H33 25,377,452 18,694,920 75 bp PE
ESC_DAXXKO_Input_H33 27,736,220 20,804,828 75 bp PE
ESC_H33WT_H3K18ac 24,126,081 19,575,195 75 bp PE
ESC_H33KO_H3K18ac 27,254,945 21,517,151 75 bp PE
ESC_H33WT_H33S31P 36,526,267 22,580,548 75 bp PE
ESC_H33WT_H33S31P_DMSO 36,368,689 15,659,365 75 bp PE
ESC_H33WT_H33S31P_Chk1i 54,998,629 26,093,156 75 bp PE
ESC_H33WT_H3K27ac_DMSO 19,853,250 15,146,812 75 bp PE
ESC_H33WT_H3K27ac_Chk1i 21,936,797 16,767,676 75 bp PE
ESC_H33WT_H3K27ac_AuroraBi 19,971,292 15,164,160 75 bp PE

Antibodies

H3.3 (09-838, Millipore, Lot # 2578126), H3K4me1 (ab8895, Abcam, Lot # GR193882-2), H3K27ac (39133, Active Motif, Lot #31814008), H3K27ac (39685, Active Motif, Lot # 14517014), H3K64ac (ABE1057, Millipore, Lot # 2826017), H3K122ac (ab33309, Abcam, Lot # GR284790-3), p300 (sc-584, Santa Cruz, Lot # F3016).

Peak calling parameters	macs2 callpeak -t $experiment -c $control -f BED -n $out_dir/$prefix -g $genomesize -p 1e-2 --mfold 10,50 --nomodel --shift 0 --extsize $fraglen --keep-dup all -B --SPMR
Data quality	FastQC tool was used to perform a quality check on all sequencing data to assess the quality of the raw reads and identify possible sequencing errors or biases to ensure that the raw data looks good and can be used for further downstream analyses. Peak calling by MACS2 reported over 70% of peaks that had above 5-fold enrichment. Enrichment metrics of pvalue 1e-2 and mfold 10,50 were used for calling peaks to ensure high-confidence enrichment against background.
Software	FastQC, BOWTIE, BWA, SAMtools, BEDtools, MACS2, R, Deeptools, Treeview