Supplementary Experimental Procedures

Chromatin Immunoprecipitations (ChIP)

hours with dox	# of cells	ug HA ab	magna beads	Volume
0	$50*10^6$	28	60 (2x30)	2x1.5ml
1	$50*10^6$	28	60 (2x30)	2x1.5ml
4	$20*10^6$	10	20	1.32ml
8	$8*10^6$	6	20	1.25ml

Number of cells used for ChIP was determined by the WB ratio of HA expression at each time-point, aiming to use equivalent amount of HA tagged protein in all samples. To avoid high background, amount of antibody was increased in proportion to the number of cells used. Cells were crosslinked with 1% formaldehyde at room temperature for 10 mins. Chromatin was extracted and then sonicated to an average size of 200–500 bp. Immunoprecipitation was carried out by using Magna ChIP™ kit as recommended by the manufacturer (Millipore) and then purified using QIAquick PCR purification kit (Qiagen). Antibodies used as positive controls (about 5 μg per 10–30 μg of DNA) were: Anti-trimethyl-Histone H3 (Lys9) (07-442, Millipore) and Anti-acetyl-Histone H3 (06-599, Millipore). IgG antibody was used as negative control (sc-2027, Santa Cruz). Amplification was carried out by real-time PCR, and the bound/input values were then normalized by setting the negative control gene results to 1.

Data Clustering

We calculated the maximal read coverage at a window of 1Kb around every annotated transcription start site (TSS), as well as the average read coverage along the gene body (starting from 500bp downstream of the TSS). We then used K-means clustering to partition the genes into four groups, using the kmeans function in MATLAB (R2014b).

Supplemental Figure legends

Supplementary Figure S1. A versatile system to study H3.3 dynamics in mouse ESCs. (A) Time course western blots showing accumulating protein levels of transgenic HA-H3.3 (upper panel) compared to GAPDH (lower panel) expression at time points from 0 to16 hours after Dox addition. (B) Quantification of the bands in Figure 1C, done by ImageJ and shown as bar graph, it should be noted that the ectopic H3.3 levels are 2-3 times higher than those of acetylated H4. (C) Live cell count of ESC with and without Dox addition for up to 72 hrs. No difference was observed in the cells survival and proliferation capacity. (D) Cells plated at different concentrations and grown for 4 days with and without RA. Live cell count shows that as expected, differentiating cells divide at a slower rate (about half) than ESC. (E) rt-qPCR results of pluripotency, selected lineage markers and housekeeping genes in KH2 ESCs and 4dRA cells, with and without Dox addition. Data are represented as log values in 4dRA relative to ESCs. (F) ChIP-PCR for H3.3-HA on KH2 ESCs and 4dRA treated cells incubated with Dox for 4H, using primers for Aprt, Esrrb, EGFR and Oct4.

Supplementary Figure S2. Gene body clustering of H3.3 dynamics correlates with expression
(A) UCSC screenshots of the indicated genes of RNA-seq and H3.3-HA or panH3 ChIP-seq data from this study (orange, green and brown density plots) and on previously published data from Deaton et al. (1) (light brown density plots), Ha et al., (2) (light green plots) and Kraushaar et al., (3) (purple plots). Boxes show TSS proximal regions. (B) Gene body heatmaps for H3.3-HA ChIP-seq on ESCs (average of 2 biological replicates). Data were clustered using maximal gene body enrichment scores. Left panel - TSS enrichment scores are shown. The right panel display the RNA-seq results of ESC without Dox or 4 hrs after Dox addition and after 4 hrs of RA treatment. RNA-seq results of ESCs without Dox addition show similar pattern. (C) TSS heatmaps for two HA-H3.3 ChIP-seq datasets, data shown as in (A) RNA-seq data is shown on the right. (D) H3.3 turnover rates in highly-expressed (green), mid-range (orange) and lowly-expressed (red) group of genes in ESCs.

Supplementary Figure S3. H3.3 turnover in bivalent genes and polycomb regions
(A) Metagene profiles of H3.3 turnover rate of genes marked only with H3K4me3 (yellow), only with H3K27me3 (blue) or of bivalent genes marked with both H3K4me3 and H3K27me3 (green). (B) Metagene plot representation of turnover rate of the TSS region of genes marked with H3K4me27 only, H3K4me3 only or both. (C) Average fold enrichment of the indicated chromatin modifications on promoters of genes from the four gene body clusters, relative to all genes analyzed (asterisks denote $P < 0.01$, hypergeometric test). (D) Average fold enrichment of Ezh2 (Ring1b negative) and Ezh2 + Ring1b (Ring1b positive) on

promoters of genes from the four gene body clusters, relative to all genes analyzed (asterisks denote P < 0.01, hypergeometric test). (E) Metagene plot of turnover rate of the TSS region of bivalent genes bound by the PRC2 member Ezh2 either with (positive) or without (negative) the PRC1 member Ring1b. (F) Metagene profiles of H3.3 turnover of bivalent genes either bound by Ring1b (positive - purple) or not bound by Ring1b (negative - light green) regions. Note significant change around the TSS.

Supplementary Figure S4: Super-enhancers proximal genes show typical turnover patterns

(A) Expression levels of genes with no enhancer, proximal to conventional enhancers or to superenhancers in ES and 4dRA confirms that superenhancers are indeed highly expressed in both cells. (B) TSS and gene body H3.3 turnover rate in ESC of genes proximal to different regulatory elements: super-enhancers, conventional enhancers and no enhancers, show a reverse correlation. (C) Average fold enrichment of different regulatory elements in ESC: super-enhancers (blue), enhancers (pink) and Oct4 binding sites (orange) on genes from the four gene-body clusters, relative to all genes. (D) Average H3.3 enrichment in a 20 Mb window 1 hr (blue), 4 hrs (gray) and 8 hrs (purple) following Dox induction around super-enhancers (left y axis). Dotted line represents average turnover rate on the right, secondary, y axis. (E) Average enrichment of canonical H3 at 1, 4, 8 hrs following DOX induction in ESC. (I) Average H3.3 enrichment in a 2 Mb window 1 hr (blue), 4 hrs (gray) and 8 hrs (purple) following Dox induction around TAD boundaries (left y axis). Dotted line on the right, secondary, y axis represents average turnover rate.

Supplementary Figure S5: Clustering is correlated with transcriptional states in ESCs

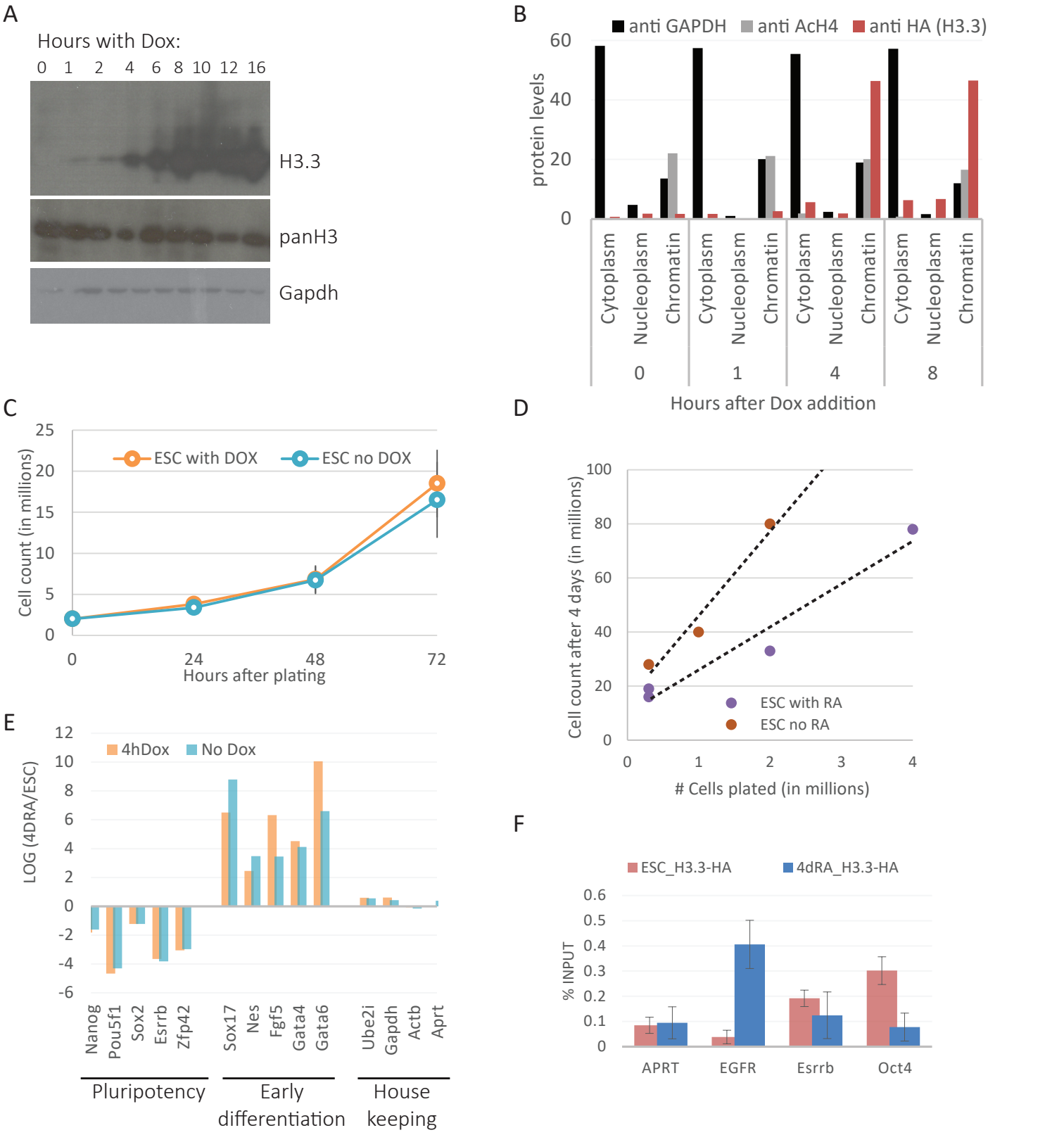
(A) Metagene profile of average turnover rate of all genes in ESCs (red, new results) and MEFs (blue, (3)) on a linear scale from TSS to TES. Turnover rate was measured using log2 of promoter HA data for 1 hr divided by 8 hrs for the ESC results and log2 of promoter HA data for 1 hr divided by 12 hrs for Kraushaar et al. Again, high values signify rapid turnover rate and low levels imply slow turnover rate. Turnover rates (in log2 scale) were normalized here to [0,1] range, to account for difference in H3.3 accumulation in the different induction systems. Similar comparisons of turnover rates of the data from Deaton et al. (1) and Ha et al. (2) were not informative due to different experimental systems (e.g. pulse vs chase). (B) Metagene averaged plot representation of previously published ChIP-seq enrichment profiles (x axis: linear scale from -5Kb to TSS, and another linear scale from TSS to TES. Y axis: read density after genome-wide normalization to 10M reads), from all time points following Dox induction. Upper left: Kraushaar et al (MEFs, GSE51505) from (3). Upper right: Ha et al (GSE63641) from (2). Lower left: Deaton et al (GSE78876) from (1) and lower right is unpublished result by us. (C) Turnover rate change between ESCs and 4dRA in gene body clusters. Aligned heatmaps are shown below. (D) Pie charts of four clusters representation of three expression levels categories (High, Mid and Low). (E) Pie

charts of four clusters representation of three differentially expressed categories (Down = expressed in ESCs, downregulated in 4dRA, UP = expression upregulated following RA treatment, no change = no change in expression following RA treatment).

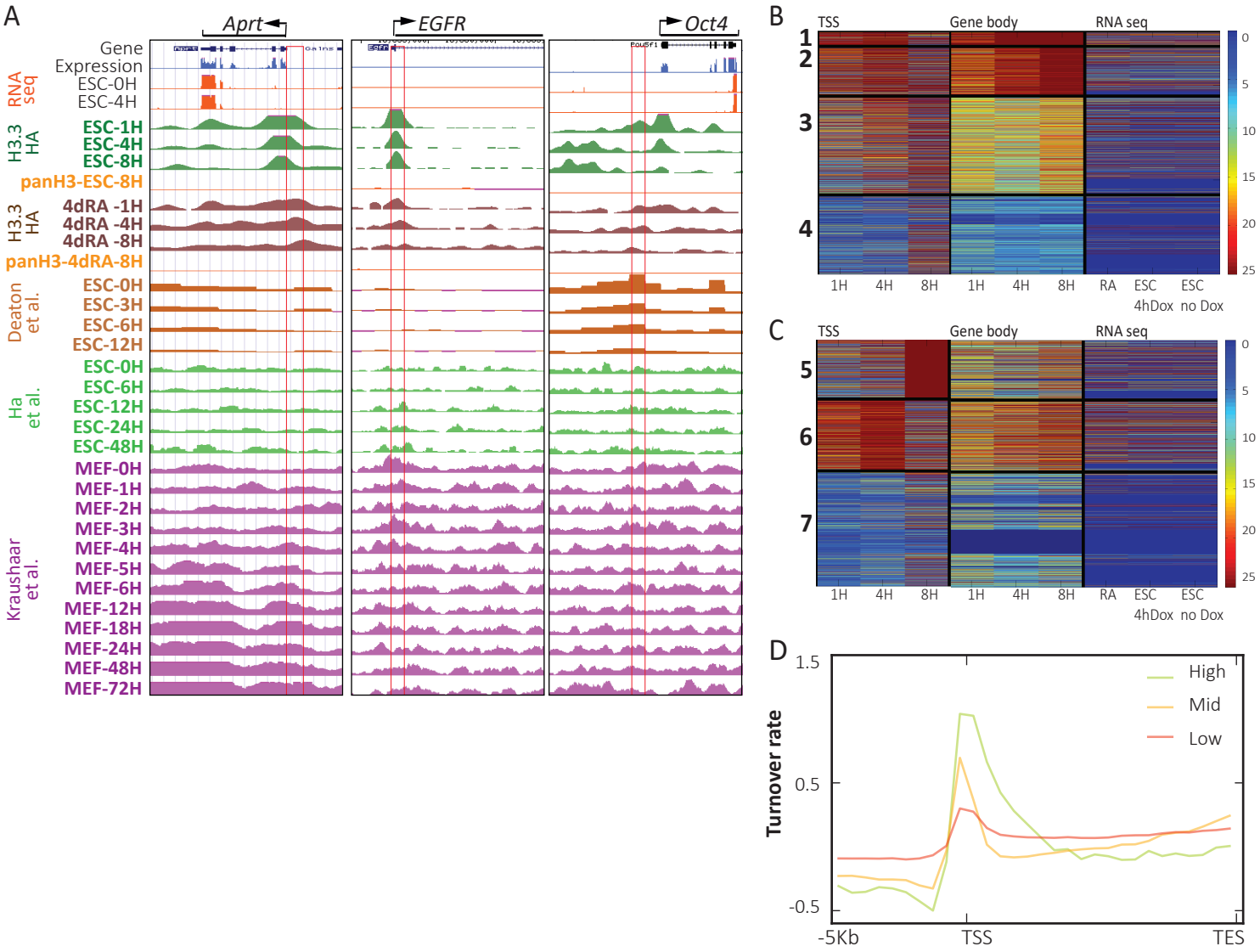
Supplemental references

1. Deaton, A.M., Gomez-Rodriguez, M., Mieczkowski, J., Tolstorukov, M.Y., Kundu, S., Sadreyev, R.I., Jansen, L.E. and Kingston, R.E. (2016) Enhancer regions show high histone H3.3 turnover that changes during differentiation. *Elife*, **5**.
2. Ha, M., Kraushaar, D.C. and Zhao, K. (2014) Genome-wide analysis of H3.3 dissociation reveals high nucleosome turnover at distal regulatory regions of embryonic stem cells. *Epigenetics Chromatin*, **7**, 38.
3. Kraushaar, D.C., Jin, W., Maunakea, A., Abraham, B., Ha, M. and Zhao, K. (2013) Genome-wide incorporation dynamics reveal distinct categories of turnover for the histone variant H3.3. *Genome Biol*, **14**, R121.

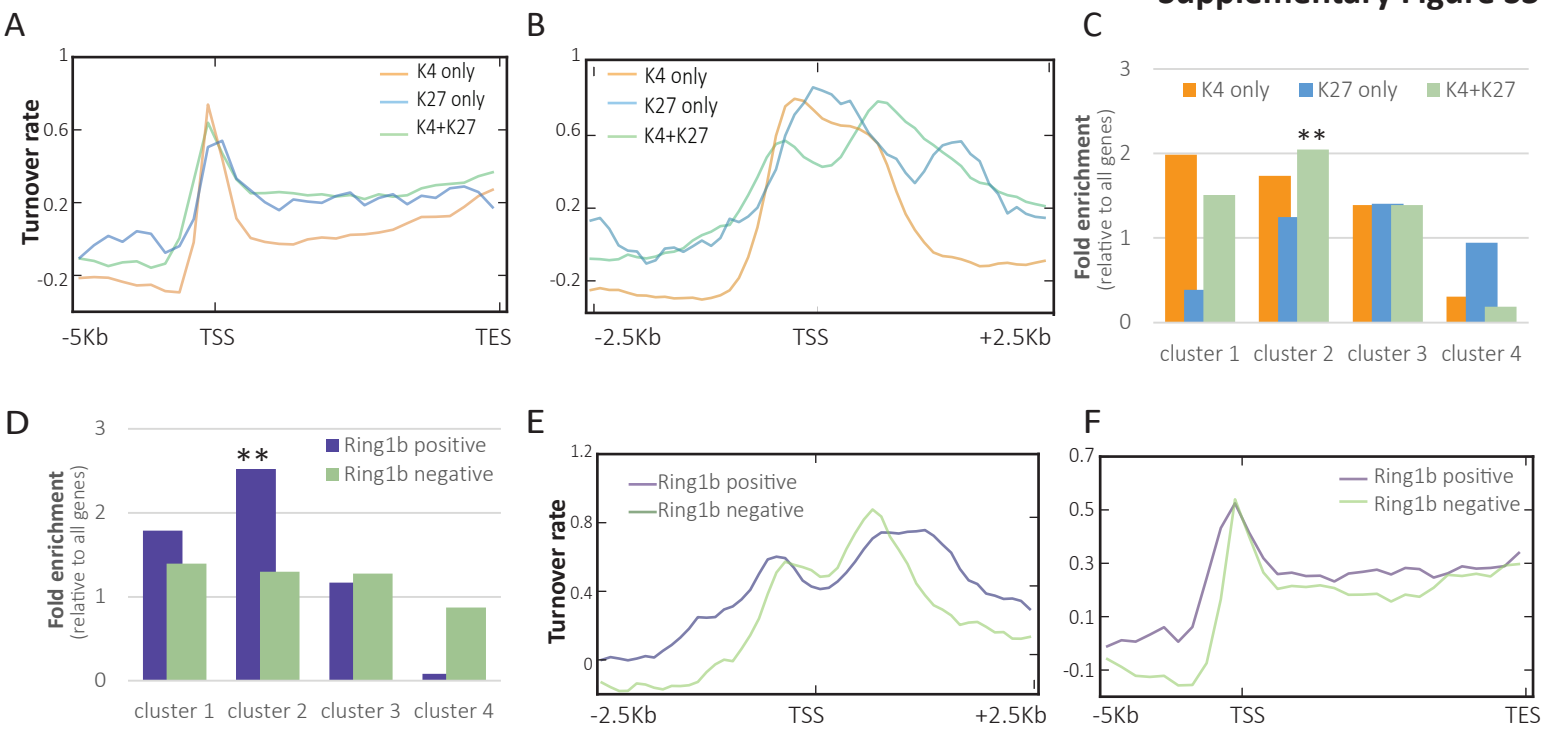
Supplementary Figure 1



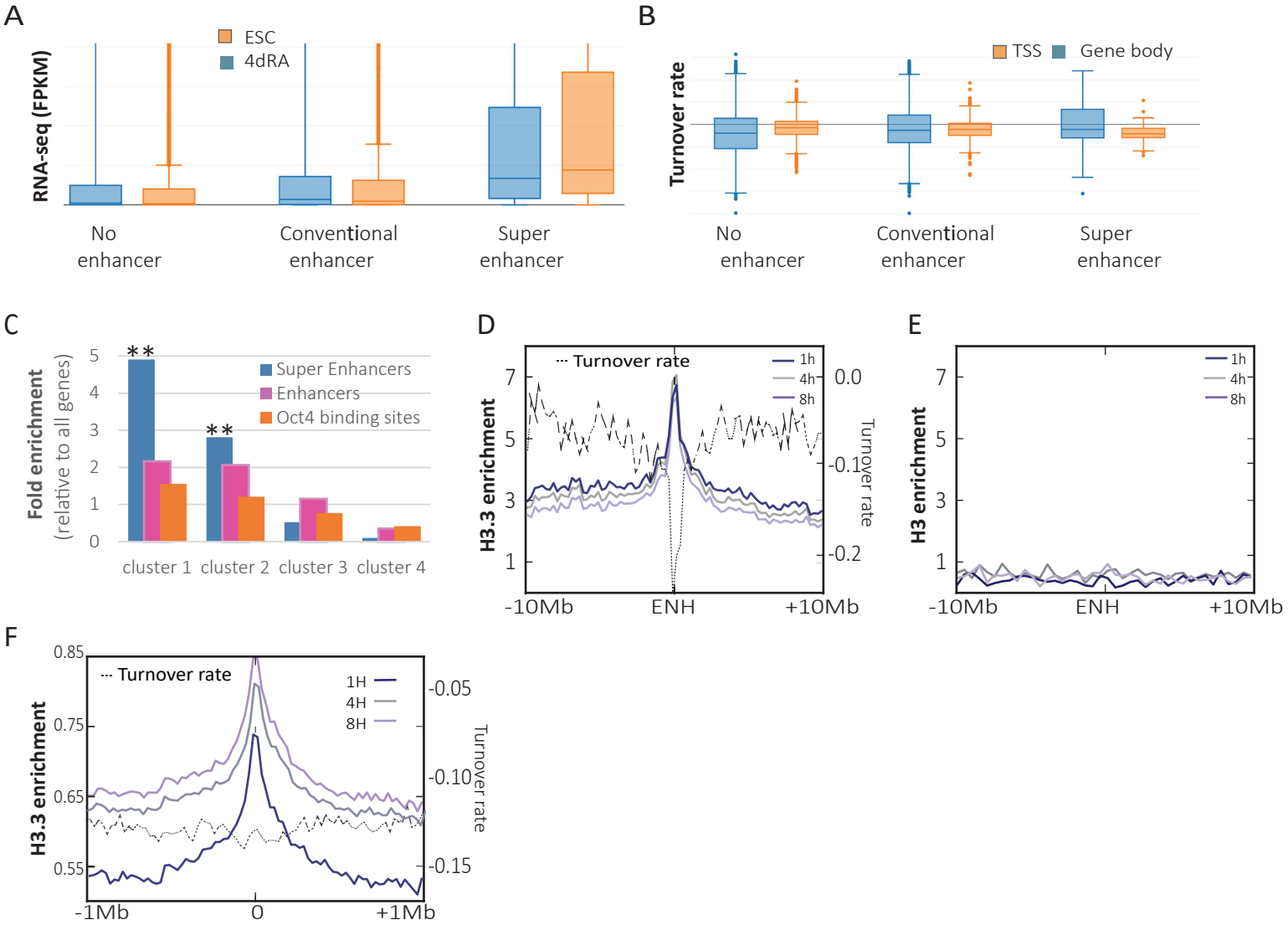
Supplementary Figure 2



Supplementary Figure S3



Supplementary Figure 4



Supplementary Figure 5