Re-evaluating the role of nucleosomal bivalency in early development

Rohan N. Shah^{1,2,*}, Adrian T. Grzybowski^{2,*}, Jimmy Elias^{2,*}, Zhonglei Chen^{3,4}, Takamitsu Hattori^{5,6}, Carolin C. Lechner^{7,8}, Peter W. Lewis⁹, Shohei Koide^{5,6,10}, Beat Fierz⁷, Alexander J. Ruthenburg^{2,10}

¹Pritzker School of Medicine, The University of Chicago, Chicago, Illinois, 60637, USA.

²Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois, 60637, USA.

³Department of Chemistry, The University of Chicago, Chicago, Illinois, 60637 USA.

⁴Present address: Lyvgen Biopharma, Shanghai 201203, P.R. China.

⁵Laura and Isaac Perlmutter Cancer Center, New York University Langone Medical Center, New York 10016, USA.

⁶Department of Biochemistry and Molecular Pharmacology, New York University School of Medicine, New York 10016, USA.

⁷Laboratory of Biophysical Chemistry of Macromolecules, Institute of Chemical Sciences and Engineering, École Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland.

⁸Present address: Bachem AG, 4416 Bubendorf, Switzerland.

⁹Wisconsin Institute for Discovery and Department of Biomolecular Chemistry, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin, USA.

¹⁰Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois, 60637, USA.

*These authors contributed equally to this work.

Correspondence and requests for materials should be addressed to A.J.R. (email: aruthenburg@uchicago.edu).

Abstract

Nucleosomes, composed of DNA and histone proteins, represent the fundamental repeating unit of the eukaryotic genome¹; posttranslational modifications of these histone proteins influence the activity of the associated genomic regions to regulate cell identity^{2–4}. Traditionally, trimethylation of histone 3-lysine 4 (H3K4me3) is associated with transcriptional initiation^{5–10}, whereas trimethylation of H3K27 (H3K27me3) is considered transcriptionally repressive^{11–15}. The apparent juxtaposition of these opposing marks, termed “bivalent domains”^{16–18}, was proposed to specifically demarcate of small set transcriptionally-poised lineage-commitment genes that resolve to one constituent modification through differentiation, thereby determining transcriptional status^{19–22}. Since then, many thousands of studies have canonized the bivalency model as a chromatin hallmark of development in many cell types. However, these conclusions are largely based on chromatin immunoprecipitations (ChIP) with significant methodological problems hampering their interpretation. Absent direct quantitative measurements, it has been difficult to evaluate the strength of the bivalency model. Here, we present reICeChIP, a calibrated sequential ChIP method to quantitatively measure H3K4me3/H3K27me3 bivalency genome-wide, addressing the limitations of prior measurements. With reICeChIP, we profile bivalency through the differentiation paradigm that first established this model^{16,18}: from naïve mouse embryonic stem cells (mESCs) into neuronal progenitor cells (NPCs). Our results cast doubt on every aspect of the bivalency model; in this context, we find that bivalency is widespread, does not resolve with differentiation, and is neither sensitive nor specific for identifying poised developmental genes or gene expression status more broadly. Our findings caution against interpreting bivalent domains as specific markers of developmentally poised genes.

In its original conception, the bivalency model posits that the combination of H3K4me3 and H3K27me3 represents a specific regulatory marker of developmentally staged genes. Specifically, lineage commitment genes are thought to be held in a low-expression, transcriptionally “poised” state by promoter nucleosomes bearing both H3K4me3 and H3K27me3^{16,18,21,22}. Upon differentiation, the bivalent domain “resolves” into a monovalent state, and the associated gene is either transcriptionally activated or terminally repressed if H3K27me3 or H3K4me3 is lost, respectively^{16,18,21,22}. The elegance of this instructive model inspired a host of follow-on studies that have suggested that bivalency is important in differentiation^{23–30}, embryogenesis^{17,31–34}, genome architecture^{22,35–38}, and oncogenesis^{39–43}.

In the absence of unambiguous biochemical or functional validation^{17,44,45}, these studies have largely relied upon ChIP, with the vast majority of studies defining loci with independent ChIP enrichment for H3K4me3 and H3K27me3 as bivalent domains. However, this analysis cannot distinguish whether the two modifications coexist or represent two distinctly marked subpopulations of alleles or cells. Further, because different ChIPs are normalized separately, they exist on separate relative scales and cannot be quantitatively compared without internal calibration^{46–48}. As such, it is impossible to quantify the extent of bivalency at a given locus or to measure its changes through differentiation.

To address the first problem, several studies have used sequential ChIP^{16,29,49–51}, measuring coexistence by using the eluent of an IP against H3K4me3 as the substrate for an IP against H3K27me3 (or vice versa). However, these experiments used antibodies of unknown specificity^{16,29,49–51}, were uncalibrated, and were often undersampled^{50,52}, precluding quantification of the extent of modification. Moreover, many used relatively large chromatin fragments in their pulldowns, making it difficult to determine whether modifications coexisted

on one nucleosome or discretely marked neighbouring nucleosomes^{16,29,50,51}. The limitations of these sequential ChIP studies preclude accurate assessment of key properties of bivalency.

Our previous work introduced internally calibrated ChIP (ICeChIP), in which barcoded nucleosome internal standards are used to measure antibody specificity and as analytical calibrants that enable computation of the histone modification density (HMD), or the proportion of nucleosomes at a given locus with the modification of interest^{46–48}. By identifying regions with high H3K4me3 and H3K27me3, we indirectly identified many promoters with a nonzero amount of bivalency, including those regulating developmental and metabolic genes⁴⁶. However, this analysis was limited; it was not sensitive for bivalency at less extensively modified loci, nor could it quantify the extent of bivalency. Here, we directly quantify this nucleosomal mark pattern by calibration of a modified sequential ChIP approach to critically evaluate the bivalency model in the differentiation system in which the foundational observations were made.

Measuring bivalency with reICeChIP

To directly measure bivalency and evaluate its role in differentiation, we first attempted to deploy our calibrants with published sequential ChIP methods. However, when evaluated with internal standards, these methods^{16,29,49} displayed extremely low enrichment and variable specificity (Extended Data Fig. 1a), with common elution methods either failing to release most of the captured material⁵¹ or compromising the specificity of the second IP (Extended Data Fig. 1b-c). With such heavy losses, we became concerned that we would undersample and potentially bias the measurement of bivalent nucleosomes. We sought a method of elution from the primary IP that was both more efficient and would preserve nucleosome integrity for the second IP. To that end, we modified a recombinant biotinylated Fab (304M3-B) specific for H3K4me3⁵³ with

an intervening HRV 3C endoprotease cleavage site to enable quantitative elution by enzymatic cleavage under mild conditions.

We then leveraged this reagent to develop reICeChIP (Fig. 1a). The first pulldown was conducted with the cleavable α -H3K4me3 Fab from native mononucleosomes^{46,54} spiked with nucleosome internal standards. We then eluted the captured nucleosomes from streptavidin resin by cleaving the antibody with HRV 3C endoprotease⁵⁵ and, with this eluent, conducted a second pulldown against H3K27me3 with a conventional antibody. This method eluted material from the primary pulldown more efficiently (Extended Data Fig. 1d), resulting in 1000-2500x higher enrichment of the target over the published methods (Fig. 1b, Extended Data Fig. 1a). This improvement enabled genome-wide measurement of bivalency HMD (Fig. 1c), representing the proportion of nucleosomes at a given locus modified with both H3K4me3 and H3K27me3, using the trans-bivalent nucleosome standards⁵⁶ as the calibrant (Extended Data Fig. 1e, 2; Supplementary Note 1).

Bivalency through differentiation

With reICeChIP, we sought to study the role of bivalency in development by tracking its changes across a differentiation pathway that was used in several classic studies of bivalency^{16,18,57}: differentiation from naïve mESCs⁵⁷ through the primed mESC state⁵⁷ to NPCs. In naïve mESCs, we noted that bivalency was far more widespread than previously reported (Fig. 2a-b); rather than ~1000 bivalent genes in naïve mESCs⁵⁷, we observed at least 10% bivalency HMD at most promoters (25768/42622), with almost 5000 promoters bearing bivalency at more than 50% of their nucleosomes (Fig. 2a,c; Supplementary Notes 2, 3). This trend is recapitulated with primed

mESCs, with the consensus set of bivalent promoters representing fewer than 2000 genes^{18,22,35,58}, as compared to more than 25,000 that are >25% bivalent in our analysis.

Even more striking were the changes in bivalency across this differentiation scheme. Previous studies suggested that bivalency largely disappears upon differentiation to NPCs^{16,18–20}. However, we found the opposite; promoter bivalency *increases* upon differentiation (Fig. 2d-e; Extended Data Fig. 3a-b), with thousands more genes meeting bivalency HMD thresholds relative to naïve mESCs (Fig. 2c). Similarly, we find that bivalent domains do not resolve upon differentiation; tracking bivalent genes from naïve mESCs through differentiation, we observe that bivalency is higher at these same promoters in primed mESCs and NPCs (Fig. 2f; Extended Data Fig. 3c-f). As previously reported, primed mESCs have the most bivalency, likely related to the high level of promoter H3K27me3 in this state⁵⁷ (Fig. 2d). Accordingly, there are 27% fewer bivalent genes in NPCs than in primed mESCs (Fig. 2e). However, this decrease is nowhere near the previously reported decrease of 92%¹⁸, and bivalent genes from primed mESCs remain highly bivalent in NPCs (Extended Data Fig. 3g-h). Collectively, these data suggest that bivalency is far more widespread in this system than previously appreciated and remains elevated through differentiation, rather than resolving to one of the two monovalent states.

To investigate this discrepancy with the literature, we compared promoters identified as bivalent by other studies^{18,20,35} to ours. The previously identified genes had 50-100% more H3K27me3 than do most bivalent genes in our set (Extended Data Fig. 4a-b), suggesting that the previous studies undersampled H3K27me3 and thus could only identify regions with high H3K27me3 as bivalent. Accordingly, H3K27me3 dominant bivalent genes had the greatest proportional overlap with these canonical bivalent loci compared to other dominance classes (i.e. whether the bivalent genes have excess H3K27me3, excess H3K4me3, or roughly equal levels as

measured by independent ICeChIP experiments for these two marks; Extended Data Fig. 4c). The common practice of measuring bivalency as regions of overlapping H3K4me3 and H3K27me3 is also problematic, even with calibrated data⁴⁶; many promoters with high H3K4me3 and H3K27me3 bear less than 25% bivalency (Extended Data Fig. 4d). Notably, even for the previously identified bivalent genes, bivalency still increases relative to naïve mESCs upon differentiation. And in our datasets, this holds true across modification dominance classes – even the H3K27me3 dominant bivalent genes, which most closely resemble the canonically bivalent loci (Extended Data Figs. 4-5). To the extent that any bivalency class resolves from naïve mESCs to NPCs, the largest set of genes is from the H3K4me3 dominant bivalent genes ($p = 1.78 \times 10^{-133}$; Fig. 2g), despite its minimal overlap with the canonical bivalent loci (Extended Data Fig. 4c).

Having found that bivalency is unexpectedly common and persistent in early differentiation, we investigated the enzyme complexes that could potentially account for this ubiquity. Previous work suggested that H3K27me3 and H3K4me3 each inhibit deposition of the other^{49,56,59,60}, particularly when symmetric (Supplementary Note 4), raising questions as to whether the pervasive bivalency we observe is plausible. To address this concern, we performed histone methyltransferase (HMTase) assays with Set1B and the full panel MLL-family core complexes (MLL1, MLL2, MLL3, MLL4), which collectively account for the bulk of H3K4 methylation in humans⁶¹. We find that these complexes all tolerate a wide spectrum of H3K27me3-decorated nucleosomes (Extended Data Fig. 6), indicating that the formation of bivalent nucleosomes is not precluded by allosteric modulation of H3K4me3 installation by core factors. Although it has been suggested that Set1a⁶², Mll2⁶³, Ezh1⁶⁴, and Ezh2⁶⁵ are all important for establishing bivalency, only Mll2 appears to be sensitive for identifying bivalent promoters in

naïve mESCs, with none showing high specificity for the same (Extended Data Fig. 7). Together, these data support the proposed specialized role for Mll2 in bivalency⁶³, indicate a pleiotropic role for PRC2 beyond its role in establishing bivalency, and provide plausible enzymatic avenues to the prevalent bivalency we observe by reICEChIP.

Bivalency, gene expression, and ontology

A key pillar of the bivalency hypothesis is that bivalent promoters are associated with transcriptionally repressed genes poised to either be activated or terminally silenced upon differentiation^{16,18,21,22}. However, bivalency is not solely found at genes with low expression in any of our measurements (Fig. 3a; Extended Data Fig. 8a-b). Rather, bivalent genes had higher average expression than did non-bivalent genes or the set of all genes, and these genes display modestly higher average expression through differentiation (Fig. 3a), with bivalency remaining similar across most gene expression deciles (Extended Data Fig. 8c). Bivalency associated similarly with bulk gene expression (Fig. 3b) and the proportion of cells expressing the associated transcripts in single cell RNA-seq (Fig. 3c), suggesting that the association of bivalency with higher-expressed genes is not solely driven by intercellular heterogeneity. Consistent with previous observations¹⁸, bivalency was higher at promoters with high CpG content (Extended Data Fig. 8d) and associated with lower DNA methylation compared to non-bivalent genes (Extended Data Fig. 8e, also holds for each dominance class). These data all suggest that bivalent genes are more highly expressed than non-bivalent genes as a whole, and this latter class is seemingly more subject to regulation by DNA methylation.

Another pillar of the bivalency model is that bivalent genes are poised to be differentially regulated through differentiation. To test this, we computed the sensitivity and specificity of

different bivalency and non-bivalency classes for differentially expressed genes (DEGs; Supplementary Note 5). Counter to the bivalency hypothesis and previous results^{16,18,21}, we found that bivalency was a very poor marker of DEGs; from naïve mESCs to NPCs, bivalency was roughly as sensitive and specific for identifying DEGs as was a *lack* of bivalency (Fig. 3d). Though H3K27me3-dominant bivalent genes showed an increase in average gene expression (Extended Data Fig. 8f-g), this class still only had 60% specificity for identifying DEGs, with very low sensitivity (Fig. 3d). Promoters of DEGs and non-DEGs from naïve mESCs to NPCs have highly similar histone modification metaprofiles in naïve mESCs (Fig. 3e-f) and across differentiation (Extended Data Fig. 8h-k). Comparison of primed mESCs to NPCs displayed similar trends (Fig. 3g-h); though sensitivity was higher because most genes are bivalent in primed mESCs, the specificity remained similar between bivalent and non-bivalent genes. Interestingly, whether genes were upregulated, downregulated, or non-DEGs, bivalency still increased over differentiation (Extended Data Fig. 8h-k). Collectively, these analyses show that bivalency is neither sensitively nor specifically associated with poised DEGs in this system.

We next examined whether bivalency is primarily associated with developmental genes, a central tenet of the original model^{16,18}. The first ICeChIP study indirectly hinted that there may be at least two classes of bivalent promoters: an H3K27me3 dominant class associated with developmental genes, and an H3K4me3 dominant class enriched for metabolic genes⁴⁶. Direct measurements of bivalency herein unambiguously demonstrate this phenomenon more broadly (Fig. 2a, 3i). Overall, bivalent genes are enriched for a broad range of ontology terms, including developmental, metabolic, and immune system process genes (Fig. 3i-j), with nearly identical bivalency profiles in naïve mESCs (Fig. 3i; Extended Data Fig. 9a). These classes all not only retained, but increased bivalency into NPCs – even immune system process genes, despite being

seemingly unrelated to neuronal development. We only found 543 genes that *did* obey the classic bivalency model (Fig. 3k), representing less than 5% of the bivalent genes from naïve mESCs, with little difference in bivalency between upregulated and downregulated genes (Extended Data Fig. 9b). Interestingly, these genes were most significantly enriched for metabolic rather than developmental genes (Fig. 3k). Taken together, these data suggest that bivalency is neither primarily nor specifically associated with developmental genes in this system.

Predicting DEGs with histone PTMs

The premise of the bivalency hypothesis is that the coexistence of H3K4me3 and H3K27me3 synergistically provides additional predictive information about the associated genes upon differentiation beyond that provided by H3K4me3 and H3K27me3 alone. With quantitative measurements of these modifications, this hypothesis can be tested by modelling. We first determined which individual parameters best identified DEGs by measuring the area under the curve (AUC) of receiver operator characteristic (ROC) curves of parameter thresholds. Of the individual histone modifications, H3K4me3 levels were best for identifying DEGs, with the highest AUC of the ROC (Fig. 4a; Extended Data Fig. 10a). Bivalency was less predictive of DEGs than were either the log ratio of H3K27me3 and H3K4me3 or DNA methylation (Fig. 4a; Extended Data Fig. 10a). And in primed mESCs, far from being predictive of poised genes, bivalency was *inversely* associated with DEGs upon differentiation to NPCs (Extended Data Fig. 10a).

If bivalency provides additional information over H3K4me3 and H3K27me3, then a model without bivalency will be markedly less explanatory than a model with bivalency. To test this, we conducted logistic regressions with linear models to identify parameters most important

for identifying DEGs. Bayes Information Criterion analyses preliminarily hinted that bivalency provided minimal information to this end (Extended Data Fig. 10b; Supplementary Note 6). To more definitively identify whether bivalency provides meaningful predictive information, we conducted hold-out cross-validation on models with H3K4me3, H3K27me3 and either nothing else, bivalency, H3K9me3, or DNA methylation (Fig. 4b; Extended Data Fig. 10c; Supplementary Note 6). Parameters other than H3K4me3 and H3K27me3 barely improved model accuracy by two separate metrics (Fig. 4c-d; Extended Data Fig. 10d-e; Supplementary Note 4), suggesting that those parameters provide virtually no additional information content to identify DEGs. These data suggest that, in this developmental system, there is little evidence that bivalency has emergent properties in identifying poised genes beyond the combined independent properties of H3K4me3 and H3K27me3.

Discussion

The bivalency hypothesis is one of the more influential ideas in epigenetics and molecular developmental biology. Persistent interest over the years coupled with widespread deployment and acceptance of sub-optimal bivalency measurement methods has ossified the hypothesis into dogma that extends well beyond any of the experimental data that informed it.

However, this coalescence has not been reached based on functional assays. Indeed, to the extent that functional validation of the bivalency model has been attempted, it has primarily been through deletion of enzymes with pleiotropic effects and functions throughout the genome beyond installation of bivalency^{35,63,66,67}. Overwhelmingly, the prevailing views on the role of bivalency are derived from ChIP experiments. However, ChIP protocols⁶⁸ and antibodies^{47,69–72} are often highly susceptible to off-target pulldown, and uncalibrated ChIP without exogenous

normalization can distort signal and the ability to compare experiments^{46,47,73}, leading to spurious conclusions⁴⁷. From the quantitative and specific measurements we made with reICEChIP, we fear that this has been the case with the bivalency hypothesis, at least as far as these analyses in early mESC differentiation permit.

It has been held that bivalency is present at a small, restricted set of promoters early in development; we find that bivalency is widespread, with many thousands of promoters displaying high bivalency levels. It has been held that bivalency primarily exists early in development and resolves upon differentiation; we find that bivalency persists at least through the NPC stage and *increases* over baseline in that span. It has been held that bivalency demarcates poised, developmental genes associated with lineage commitment; we find that bivalency is neither sensitively nor specifically associated with developmental nor differentially expressed genes – and, at worst, may be *inversely* associated with the latter. Moreover, bivalent genes are predominantly not poised in an off state, but are more highly expressed than those that are not bivalent. All told, we find little evidence that bivalency provides more information in predicting poised gene status than do H3K4me3 and H3K27me3 in an independently additive manner in this system, raising questions as to whether it represents any more than a coincidental overlap of the aforementioned two marks.

Our study is not without caveats. First, we are only able to comment meaningfully on the differentiation paradigm presented here; we cannot definitively infer that these results will hold for the other developmental or clinical contexts. Although the original studies on bivalency indicated that bivalency almost entirely disappeared by the NPC stage^{16,18}, this stage is not terminally differentiated, so it is possible that bivalency could resolve in later stages of differentiation. Future studies will be needed to address this possibility in other developmental

contexts. Second, though the extant evidence suggests that only trans-bivalency is present at meaningful levels, our method cannot selectively distinguish between *cis*-, *trans*-, and intermediate bivalency conformations (Supplementary Note 1).

The reICeChIP method is not inherently restricted to the study of H3K4me3/H3K27me3 bivalency. With cleavable recombinant affinity reagents targeting other histone modifications^{53,74} it could be used to quantify other combinatorial modification patterns^{75–78} or modification symmetry.

Without serious changes to the standards of ChIP, the limitations of conventional ChIP-seq will continue to pose an existential challenge to the field. Indeed, the divergence between our observations of bivalency and those in the literature can be attributed to the historical lack of tools needed to make quantitative and specific measurements; in that context, the experimental designs and interpretations of the past were reasonable. Fortunately, such tools now exist. And as we have shown in this work, these methods offer a chance for the field to critically evaluate its orthodox models and pave the way for new insights on the chromatin determinants of cell identity and the regulation of development.

Figure Captions

Fig. 1 | Workflow and evaluation of reICeChIP-seq. (a) Schematic of reICeChIP-seq. The recombinant α -H3K4me3 Fab 304M3-B achieves high affinity by "clasping" the histone tail between two Fab molecules⁵³, a binding mode readily achieved by multiple copies of the Fab presented on a bead, but not by the Fab in solution. Thus, protease cleavage not only elutes nucleosomes from the beads but also likely from the Fab complex. (b) Enrichment of different barcoded nucleosomes in reICeChIP-seq (n=3 biological replicates). Error bars represent S.D. (c) Representative line plot showing histone modification density of H3K4me3, H3K27me3, and bivalency ICeChIP-seq presented with 95% confidence intervals (lighter shade) and input read depth in naïve mESCs. Bivalency is calibrated to the trans-bivalency nucleosome standard and corrected for off-target H3K9me3 pulldown.

Fig. 2 | Bivalency is widespread and does not resolve over differentiation. (a) Bivalency, H3K4me3, and H3K27me3 at all Refseq promoters in naïve mESCs, with relative enrichment of GO terms. Genes are rank ordered by bivalency HMD at promoter, defined as the region from 0 to +400 bp relative to the TSS. (b) Representative locus view of H3K4me3, H3K27me3, and bivalency at promoters in naïve mESCs (top), primed mESCs (centre), and NPCs (bottom), presented on the same scale of 0-125% HMD. (c) Number of promoters with bivalency HMDs above the given thresholds in each cell type out of a total of 42,622 Refseq promoters. (d) Metaprofiles of H3K4me3, H3K27me3, and bivalency at all promoters in naïve mESCs, primed mESCs, and NPCs. Heatmaps for primed mESCs and NPCs are presented in Extended Data Fig. 3b. (e) Distribution of bivalency HMDs at all Refseq promoters in three cell states, zoomed to below 125% HMD. Overall, 99.5% of naïve promoters, 87.3% of primed promoters, and 91.6%

of NPC promoters have an HMD below 100%. Full plot in Extended Data Fig. 3a. **(f)** Metaprofiles of H3K4me3, H3K27me3, and bivalency at promoters identified as bivalent in naïve mESCs (25% HMD threshold), tracked from naïve mESCs to primed mESCs to NPCs. Heatmaps for bivalency are presented in Extended Data Fig. 3f. **(g-h)** Alluvial plots of dominance and bivalency of genes from (g) naïve mESCs to NPCs or (h) primed mESCs to NPCs. Bivalency [$>25\%$ HMD] can be subcategorized into dominance classes by independent ICeChIP for the constituent marks, with H3K27me3 in excess ($\text{H3K27me3}/\text{H3K4me3} > e^1$), H3K4me3 dominant ($\text{H3K27me3}/\text{H3K4me3} < e^{-1}$), or intermediate ratios (no dominance). **(i)** Bivalency metaprofiles for gene subsets indicated in panel (h) from -3kb to +3kb relative to the TSS. **** $p < 2.2 \times 10^{-16}$.

Fig. 3 | Bivalency is neither sensitive nor specific for poised nor developmental genes. (a)

Violin plots of gene expression⁷⁹ for all genes in naïve mESCs, non-bivalent genes ($<25\%$ HMD) in naïve mESCs, and bivalent genes ($>25\%$ HMD) tracked from naïve mESCs to the same genes in the indicated lineages. Significance computed by Welch's two-tailed t -test. **(b)** Gene expression vs. HMD for H3K4me3, H3K27me3, and bivalency (genes are binned into HMD deciles). **(c)** Proportion of actively transcribing cells by single-cell RNA-seq⁸⁰ vs. HMD for H3K4me3, H3K27me3, and bivalency (genes are binned into HMD deciles). **(d)** Sensitivity and specificity (Supplementary Note 5) of bivalent and non-bivalent genes in naïve mESCs identifying differentially expressed genes (DEGs) from the naïve state to the NPC state. **(e)** Metaprofiles of H3K4me3, H3K27me3, and bivalency and **(f)** heatmaps of bivalency in naïve mESCs at DEGs and non-DEGs relative to NPCs. **(g)** Sensitivity and specificity of bivalent and non-bivalent genes in primed mESCs identifying DEGs from the primed state to the NPC state.

(h) Metaprofiles of H3K4me3, H3K27me3, and bivalency in primed mESCs at DEGs and non-DEGs. (i) Gene ontology term enrichment of H3K27me3-dominant bivalent genes, H3K4me3-dominant bivalent genes, or bivalent genes with no clear dominance (q-value two-tailed Fisher hypergeometric test). (j) Metaprofiles of H3K4me3, H3K27me3, and bivalency in naïve mESCs at developmental and metabolic genes. (k) Gene ontology term enrichment of genes following the classic bivalency model: DEGs that lose bivalency from naïve mESCs (>25% HMD) to NPCs (<10% HMD). Significance computed by two-tailed Fisher hypergeometric test. * $q < 0.05$. ** $q < 0.01$. **** p or $q < 2.2 \times 10^{-16}$.

Fig. 4 | Bivalency does not provide appreciably more information than H3K4me3 and H3K27me3 alone for DEG prediction. (a) Receiver operator characteristic (ROC) curves for identifying DEGs from naïve mESCs to NPCs by H3K4me3, H3K9me3, H3K27me3, bivalency, $\ln(\text{H3K27me3}/\text{H3K4me3})$, or DNA methylation in naïve mESCs. For each point, parameter value threshold used to compute true positive rate (TPR) and false positive rate (FPR) is indicated by the colour. Traits with thresholds identifying non-DEGs rather than DEGs are marked with “rev.” (b) Legend for generalized linear models (GLMs) in panels c-d. (c) Accuracy of trivial model and GLMs by threshold accuracy (gene identified as DEG if logistic regression > 0.5; left) and by ROC area under curve (right). (d) ROC curves for identifying DEGs from naïve mESCs to NPCs by different GLMs. For each point, logistic regression threshold value used to compute TPR and FPR is indicated by the colour.

References

1. Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F. & Richmond, T. J. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389**, 18 (1997).
2. Strahl, B. D. & Allis, C. D. The language of covalent histone modifications. *Nature* **403**, 41–45 (2000).
3. Kouzarides, T. Chromatin Modifications and Their Function. *Cell* **128**, 693–705 (2007).
4. Bannister, A. J. & Kouzarides, T. Regulation of chromatin by histone modifications. *Cell Res.* **21**, 381–395 (2011).
5. Santos-Rosa, H. *et al.* Active genes are tri-methylated at K4 of histone H3. *Nature* **419**, 407–411 (2002).
6. Wysocka, J. *et al.* A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature* **442**, 86–90 (2006).
7. Heintzman, N. D. *et al.* Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat. Genet.* **39**, 311–318 (2007).
8. Guenther, M. G., Levine, S. S., Boyer, L. A., Jaenisch, R. & Young, R. A. A Chromatin Landmark and Transcription Initiation at Most Promoters in Human Cells. *Cell* **130**, 77–88 (2007).
9. Vermeulen, M. *et al.* Selective Anchoring of TFIID to Nucleosomes by Trimethylation of Histone H3 Lysine 4. *Cell* **131**, 58–69 (2007).
10. Lauberth, S. M. *et al.* H3K4me3 Interactions with TAF3 Regulate Preinitiation Complex Assembly and Selective Gene Activation. *Cell* **152**, 1021–1036 (2013).

- 947 11. Ringrose, L., Ehret, H. & Paro, R. Distinct Contributions of Histone H3 Lysine 9 and 27
948 Methylation to Locus-Specific Stability of Polycomb Complexes. *Mol. Cell* **16**, 641–653 (2004).
- 949 12. Boyer, L. A. *et al.* Polycomb complexes repress developmental regulators in murine
950 embryonic stem cells. *Nature* **441**, 349–353 (2006).
- 951 13. O’Carroll, D. *et al.* The Polycomb-Group Gene *Ezh2* Is Required for Early Mouse
952 Development. *Mol. Cell. Biol.* **21**, 4330–4336 (2001).
- 953 14. Pasini, D., Bracken, A. P., Jensen, M. R., Lazzerini Denchi, E. & Helin, K. Suz12 is
954 essential for mouse development and for EZH2 histone methyltransferase activity. *EMBO J.* **23**,
955 4061–4071 (2004).
- 956 15. Pasini, D., Bracken, A. P., Hansen, J. B., Capillo, M. & Helin, K. The Polycomb Group
957 Protein Suz12 Is Required for Embryonic Stem Cell Differentiation. *Mol. Cell. Biol.* **27**, 3769–
958 3779 (2007).
- 959 16. Bernstein, B. E. *et al.* A Bivalent Chromatin Structure Marks Key Developmental Genes
960 in Embryonic Stem Cells. *Cell* **125**, 315–326 (2006).
- 961 17. Azuara, V. *et al.* Chromatin signatures of pluripotent cell lines. *Nat. Cell Biol.* **8**, 532–
962 538 (2006).
- 963 18. Mikkelsen, T. S. *et al.* Genome-wide maps of chromatin state in pluripotent and lineage-
964 committed cells. *Nature* **448**, 553–560 (2007).
- 965 19. Gifford, C. A. *et al.* Transcriptional and Epigenetic Dynamics during Specification of
966 Human Embryonic Stem Cells. *Cell* **153**, 1149–1163 (2013).
- 967 20. Xie, W. *et al.* Epigenomic Analysis of Multilineage Differentiation of Human Embryonic
968 Stem Cells. *Cell* **153**, 1134–1148 (2013).

- 969 21. Voigt, P., Tee, W.-W. & Reinberg, D. A double take on bivalent promoters. *Genes Dev.*
970 **27**, 1318–1338 (2013).
- 971 22. Blanco, E., González-Ramírez, M., Alcaine-Colet, A., Aranda, S. & Di Croce, L. The
972 Bivalent Genome: Characterization, Structure, and Regulation. *Trends Genet.* **36**, 118–131
973 (2020).
- 974 23. Kanayama, K. *et al.* Genome-Wide Mapping of Bivalent Histone Modifications in
975 Hepatic Stem/Progenitor Cells. *Stem Cells Int.* **2019**, e9789240 (2019).
- 976 24. Zhou, Y. *et al.* Bivalent Histone Codes on WNT5A during Odontogenic Differentiation.
977 *J. Dent. Res.* **97**, 99–107 (2018).
- 978 25. Burney, M. J. *et al.* An epigenetic signature of developmental potential in neural stem
979 cells and early neurons. *STEM CELLS* **31**, 1868–1880 (2013).
- 980 26. Tanimura, K., Suzuki, T., Vargas, D., Shibata, H. & Inagaki, T. Epigenetic regulation of
981 beige adipocyte fate by histone methylation. *Endocr. J.* **66**, 115–125 (2019).
- 982 27. Dhar, S. S. *et al.* An essential role for UTX in resolution and activation of bivalent
983 promoters. *Nucleic Acids Res.* **44**, 3659–3674 (2016).
- 984 28. Abraham, B. J., Cui, K., Tang, Q. & Zhao, K. Dynamic regulation of epigenomic
985 landscapes during hematopoiesis. *BMC Genomics* **14**, 193 (2013).
- 986 29. Seenundun, S. *et al.* UTX mediates demethylation of H3K27me3 at muscle-specific
987 genes during myogenesis. *EMBO J.* **29**, 1401–1411 (2010).
- 988 30. Cui, K. *et al.* Chromatin Signatures in Multipotent Human Hematopoietic Stem Cells
989 Indicate the Fate of Bivalent Genes during Differentiation. *Cell Stem Cell* **4**, 80–93 (2009).

- 990 31. Dahl, J. A., Reiner, A. H., Klungland, A., Wakayama, T. & Collas, P. Histone H3 Lysine
991 27 Methylation Asymmetry on Developmentally-Regulated Promoters Distinguish the First Two
992 Lineages in Mouse Preimplantation Embryos. *PLOS ONE* **5**, e9150 (2010).
- 993 32. Vastenhouw, N. L. *et al.* Chromatin signature of embryonic pluripotency is established
994 during genome activation. *Nature* **464**, 922–926 (2010).
- 995 33. Vastenhouw, N. L. & Schier, A. F. Bivalent histone modifications in early
996 embryogenesis. *Curr. Opin. Cell Biol.* **24**, 374–386 (2012).
- 997 34. Alder, O. *et al.* Ring1B and Suv39h1 delineate distinct chromatin states at bivalent genes
998 during early mouse lineage commitment. *Development* **137**, 2483–2492 (2010).
- 999 35. Mas, G. *et al.* Promoter bivalency favors an open chromatin architecture in embryonic
1000 stem cells. *Nat. Genet.* **50**, 1452–1462 (2018).
- 1001 36. Denholtz, M. *et al.* Long-Range Chromatin Contacts in Embryonic Stem Cells Reveal a
1002 Role for Pluripotency Factors and Polycomb Proteins in Genome Organization. *Cell Stem Cell*
1003 **13**, 602–616 (2013).
- 1004 37. Vieux-Rochas, M., Fabre, P. J., Leleu, M., Duboule, D. & Noordermeer, D. Clustering of
1005 mammalian Hox genes with other H3K27me3 targets within an active nuclear domain. *Proc.*
1006 *Natl. Acad. Sci.* **112**, 4672–4677 (2015).
- 1007 38. Kundu, S. *et al.* Polycomb Repressive Complex 1 Generates Discrete Compacted
1008 Domains that Change during Differentiation. *Mol. Cell* **65**, 432-446.e5 (2017).
- 1009 39. Messier, T. L. *et al.* Oncofetal Epigenetic Bivalency in Breast Cancer Cells: H3K4 and
1010 H3K27 Tri-Methylation as a Biomarker for Phenotypic Plasticity. *J. Cell. Physiol.* **231**, 2474–
1011 2481 (2016).

- 1012 40. Kaukonen, D. *et al.* Analysis of H3K4me3 and H3K27me3 bivalent promoters in HER2+
1013 breast cancer cell lines reveals variations depending on estrogen receptor status and significantly
1014 correlates with gene expression. *BMC Med. Genomics* **13**, 92 (2020).
- 1015 41. Dunican, D. S. *et al.* Bivalent promoter hypermethylation in cancer is linked to the
1016 H327me3/H3K4me3 ratio in embryonic stem cells. *BMC Biol.* **18**, 25 (2020).
- 1017 42. Burr, M. L. *et al.* An Evolutionarily Conserved Function of Polycomb Silences the MHC
1018 Class I Antigen Presentation Pathway and Enables Immune Evasion in Cancer. *Cancer Cell* **36**,
1019 385-401.e8 (2019).
- 1020 43. Patani, H. *et al.* Transition to naïve human pluripotency mirrors pan-cancer DNA
1021 hypermethylation. *Nat. Commun.* **11**, 3671 (2020).
- 1022 44. Hu, D. *et al.* The Mll2 branch of the COMPASS family regulates bivalent promoters in
1023 mouse embryonic stem cells. *Nat. Struct. Mol. Biol.* **20**, 1093–1097 (2013).
- 1024 45. Hu, D. *et al.* Not All H3K4 Methylations Are Created Equal: Mll2/COMPASS
1025 Dependency in Primordial Germ Cell Specification. *Mol. Cell* **65**, 460-475.e6 (2017).
- 1026 46. Grzybowski, A. T., Chen, Z. & Ruthenburg, A. J. Calibrating ChIP-Seq with
1027 Nucleosomal Internal Standards to Measure Histone Modification Density Genome Wide. *Mol.*
1028 *Cell* **58**, 886–899 (2015).
- 1029 47. Shah, R. N. *et al.* Examining the Roles of H3K4 Methylation States with Systematically
1030 Characterized Antibodies. *Mol. Cell* **72**, 162–177 (2018).
- 1031 48. Grzybowski, A. T., Shah, R. N., Richter, W. F. & Ruthenburg, A. J. Native internally
1032 calibrated chromatin immunoprecipitation for quantitative studies of histone post-translational
1033 modifications. *Nat. Protoc.* **14**, 3275–3302 (2019).

- 1034 49. Voigt, P. *et al.* Asymmetrically Modified Nucleosomes. *Cell* **151**, 181–193 (2012).
- 1035 50. Weiner, A. *et al.* Co-ChIP enables genome-wide mapping of histone mark co-occurrence
1036 at single-molecule resolution. *Nat. Biotechnol.* **34**, 953–961 (2016).
- 1037 51. Kinkley, S. *et al.* reChIP-seq reveals widespread bivalency of H3K4me3 and H3K27me3
1038 in CD4⁺ memory T cells. *Nat. Commun.* **7**, 12514 (2016).
- 1039 52. Shema, E. *et al.* Single-molecule decoding of combinatorially modified nucleosomes.
1040 *Science* **352**, 717–721 (2016).
- 1041 53. Hattori, T. *et al.* Antigen clasping by two antigen-binding sites of an exceptionally
1042 specific antibody for histone methylation. *Proc. Natl. Acad. Sci.* **113**, 2092–2097 (2016).
- 1043 54. Brand, M., Rampalli, S., Chaturvedi, C.-P. & Dilworth, F. J. Analysis of epigenetic
1044 modifications of chromatin at specific gene loci by native chromatin immunoprecipitation of
1045 nucleosomes isolated using hydroxyapatite chromatography. *Nat. Protoc.* **3**, 398–409 (2008).
- 1046 55. Raran-Kurussi, S., Tözsér, J., Cherry, S., Tropea, J. E. & Waugh, D. S. Differential
1047 Temperature Dependence of Tobacco Etch Virus and Rhinovirus 3C Proteases. *Anal. Biochem.*
1048 **436**, 142–144 (2013).
- 1049 56. Lechner, C. C., Agashe, N. D. & Fierz, B. Traceless Synthesis of Asymmetrically
1050 Modified Bivalent Nucleosomes. *Angew. Chem. Int. Ed.* **55**, 2903–2906 (2016).
- 1051 57. Marks, H. *et al.* The Transcriptional and Epigenomic Foundations of Ground State
1052 Pluripotency. *Cell* **149**, 590–604 (2012).
- 1053 58. Xiao, S. *et al.* Comparative Epigenomic Annotation of Regulatory DNA. *Cell* **149**, 1381–
1054 1392 (2012).

- 1055 59. Schmitges, F. W. *et al.* Histone Methylation by PRC2 Is Inhibited by Active Chromatin
1056 Marks. *Mol. Cell* **42**, 330–341 (2011).
- 1057 60. Kim, D.-H. *et al.* Histone H3K27 Trimethylation Inhibits H3 Binding and Function of
1058 SET1-Like H3K4 Methyltransferase Complexes. *Mol. Cell. Biol.* **33**, 4936–4946 (2013).
- 1059 61. Shilatifard, A. The COMPASS Family of Histone H3K4 Methylases: Mechanisms of
1060 Regulation in Development and Disease Pathogenesis. *Annu. Rev. Biochem.* **81**, 65–95 (2012).
- 1061 62. Sze, C. C. *et al.* Histone H3K4 methylation-dependent and -independent functions of
1062 Set1A/COMPASS in embryonic stem cell self-renewal and differentiation. *Genes Dev.* **31**,
1063 1732–1737 (2017).
- 1064 63. Denissov, S. *et al.* Mll2 is required for H3K4 trimethylation on bivalent promoters in
1065 embryonic stem cells, whereas Mll1 is redundant. *Development* **141**, 526–537 (2014).
- 1066 64. Aoyama, K. *et al.* Ezh1 Targets Bivalent Genes to Maintain Self-Renewing Stem Cells in
1067 Ezh2-Insufficient Myelodysplastic Syndrome. *iScience* **9**, 161–174 (2018).
- 1068 65. Béguelin, W. *et al.* EZH2 and BCL6 Cooperate to Assemble CBX8-BCOR Complex to
1069 Repress Bivalent Promoters, Mediate Germinal Center Formation and Lymphomagenesis.
1070 *Cancer Cell* **30**, 197–213 (2016).
- 1071 66. Tan, H. K. *et al.* DNMT3B shapes the mCA landscape and regulates mCG for promoter
1072 bivalency in human embryonic stem cells. *Nucleic Acids Res.* **47**, 7460–7475 (2019).
- 1073 67. Eckersley-Maslin, M. A. *et al.* Epigenetic priming by Dppa2 and 4 in pluripotency
1074 facilitates multi-lineage commitment. *Nat. Struct. Mol. Biol.* **27**, 696–705 (2020).

- 1075 68. Kasinathan, S., Orsi, G. A., Zentner, G. E., Ahmad, K. & Henikoff, S. High-resolution
1076 mapping of transcription factor binding sites on native chromatin. *Nat. Methods* **11**, 203–209
1077 (2014).
- 1078 69. Bock, I. *et al.* Detailed specificity analysis of antibodies binding to modified histone tails
1079 with peptide arrays. *Epigenetics* **6**, 256–263 (2011).
- 1080 70. Egelhofer, T. A. *et al.* An assessment of histone-modification antibody quality. *Nat.*
1081 *Struct. Mol. Biol.* **18**, 91–93 (2011).
- 1082 71. Nishikori, S. *et al.* Broad Ranges of Affinity and Specificity of Anti-Histone Antibodies
1083 Revealed by a Quantitative Peptide Immunoprecipitation Assay. *J. Mol. Biol.* **424**, 391–399
1084 (2012).
- 1085 72. Fuchs, S. M., Krajewski, K., Baker, R. W., Miller, V. L. & Strahl, B. D. Influence of
1086 Combinatorial Histone Modifications on Antibody and Effector Protein Recognition. *Curr. Biol.*
1087 **21**, 53–58 (2011).
- 1088 73. Orlando, D. A. *et al.* Quantitative ChIP-Seq Normalization Reveals Global Modulation of
1089 the Epigenome. *Cell Rep.* **9**, 1163–1170 (2014).
- 1090 74. Hattori, T. *et al.* Recombinant antibodies to histone post-translational modifications. *Nat.*
1091 *Methods* **10**, 992–995 (2013).
- 1092 75. Allis, C. D. & Jenuwein, T. The molecular hallmarks of epigenetic control. *Nat. Rev.*
1093 *Genet.* **17**, 487–500 (2016).
- 1094 76. Lu, C., Coradin, M., Janssen, K. A., Sidoli, S. & Garcia, B. A. Combinatorial Histone H3
1095 Modifications Are Dynamically Altered in Distinct Cell Cycle Phases. *J. Am. Soc. Mass*
1096 *Spectrom.* **32**, 1300–1311 (2021).

1097 77. Suganuma, T. & Workman, J. L. Signals and Combinatorial Functions of Histone
1098 Modifications. *Annu. Rev. Biochem.* **80**, 473–499 (2011).

1099 78. Henikoff, S. Histone modifications: Combinatorial complexity or cumulative simplicity?
1100 *Proc. Natl. Acad. Sci.* **102**, 5308–5309 (2005).

1101 79. Terranova, C. *et al.* Global Developmental Gene Programing Involves a Nuclear Form of
1102 Fibroblast Growth Factor Receptor-1 (FGFR1). *PLOS ONE* **10**, e0123380 (2015).

1103 80. Xing, Q. R. *et al.* Parallel bimodal single-cell sequencing of transcriptome and chromatin
1104 accessibility. *Genome Res.* **30**, 1027–1039 (2020).

1105

Acknowledgements

We would like to thank Peter Faber, Hannah Whitehurst, and Mikayla Marchuk in the University of Chicago Functional Genomics Facility for Illumina sequencing. We would also like to thank EpiCypher, Inc. for providing some of the histone octamers for this study. A.T.G. was supported by the Harper Dissertation Prize and the Dean's International Student Fellowship of the University of Chicago. R.N.S. was supported by the National Institutes of Health under award number T32-HD007009-45 to the University of Chicago. J. E. was supported by the National Institutes of Health under award number T32-GM007197 and R25-GM109439 to the University of Chicago. This study was supported by the National Institutes of Health, under award numbers R01-GM115945 to A.J.R. and R01-DA036887 to S.K.; and the American Cancer Society, under award number 130230-RSG-16-248-01-DMC to A.J.R.

Author Contributions

A.T.G. and A.J.R. conceived of the study. Z.C. made *cis*-bivalent H3K4me3-H3K27me3 histones. C.C.L. and B.F. constructed the *trans*-bivalent H3K4me3-H3K27me3 histone dimers. T.H. and S.K. provided 304M3B Fab and assisted in design of 304M3B-1HRV3C Fab. P.W.L. provided recombinant PRC2 for supporting experiments. A.T.G. developed reICeChIP, expressed and purified 304M3B-1HRV3C Fab, and conducted methyltransferase assays with input and oversight from A.J.R. A.T.G. cultured naïve mESCs; J.E. cultured and differentiated naïve mESCs to primed mESCs and NPCs. A.T.G. conducted reICeChIP-seq on naïve mESCs; R.N.S. conducted reICeChIP-seq on primed mESCs and NPCs. A.T.G. and R.N.S. conducted bioinformatic analysis on naïve mESCs; J.E. generated representative genome browser views. R.N.S. conducted all other bioinformatic analyses with oversight and input from A.T.G., J.E.,

1193 and A.J.R. R.N.S., A.T.G., J.E., and A.J.R. wrote the manuscript with input from the other
1194 authors.

1195

1196 **Competing Interests**

1197 The authors declare competing financial interests. A.T.G., Z.C. and A.J.R hold partial
1198 intellectual property rights to ICeChIP as co-inventors on a patent filed by the University of
1199 Chicago [Patent # US20200319204A1]). This patent is under license to EpiCypher, Inc., a
1200 commercial developer and supplier of platforms similar to ICeChIP with barcoded nucleosomes
1201 (i.e. SNAP-ChIPTM and CAP-ChIPTM). R.N.S., A.T.G., and A.J.R. have served in a compensated
1202 consulting role to EpiCypher, Inc., and A.J.R. is a member of the Scientific Advisory Board.

1203

1204 **Additional Information**

1205 **Supplementary Information** is available for this paper.

1206 **Correspondence and requests for materials** should be addressed to A.J.R.

1207 **Reprints and permissions information** is available at <http://www.nature.com/reprints>.

Extended Data Figure Captions

Extended Data Fig. 1 | Evaluation of sequential ChIP methods. (a) Enrichment of on- and off-target nucleosome standards under sequential ChIP protocols developed by Bernstein et al.¹⁶, Voigt et al.⁴⁹, and Seenundun et al.²⁹. (b-c) Enrichment at different sequential ICeChIP steps with (b) chemical denaturant elution and (c) immunoglobulin and serum elution. (d) Enrichment of different nucleosome standards with ICeChIP-qPCR performed against H3K4me3, H3K27me3, and bivalency, with beads showing very little retention of chromatin (n=3 technical replicates). Error bars represent standard deviation. (e) Different configurations of bivalency on a single nucleosome. Of these, only trans-bivalency has been identified by mass spectrometry^{49,81}.

Extended Data Fig. 2 | Evaluation of reICeChIP specificity and standards. (a) Representative genome browser view of H3K4me3, H3K27me3, and bivalency, shown as a range of possible values by normalization to trans-bivalent (upper limit) or cis-bivalent (lower limit) nucleosome standards. (b) Relative pulldown of different nucleosome standards in ICeChIP-seq, normalized to the most-enriched standard. (c) Scatterplots of reads from DNA barcodes applied to nucleosome standards in ICeChIP-seq. (d) Violin plots of peak breadth (consecutive segment of 50bp windows overlapping promoter with >25% HMD) for H3K4me3 (green) and bivalency (blue) at non-bivalent and bivalent genes (>25% HMD) in naïve mESCs. (e-f) Autocorrelation of (e) H3K4me3 and (f) bivalency HMDs between nucleosomes in naïve mESCs. Nucleosomes are defined as sequential 200bp windows from the TSS.

Extended Data Fig. 3 | Tracking bivalent genes through differentiation. (a) Distribution of bivalency HMDs at all Refseq promoters in three cell states. (b) Heatmaps of bivalency at all Refseq promoters in primed mESCs and NPCs. Genes are ordered by bivalency HMD at the promoter. (c) Venn diagram showing overlap of bivalent genes (25% HMD threshold) in naïve mESCs, primed mESCs, and NPCs. (d-e) Metaprofiles of H3K4me3, H3K27me3, and bivalency for bivalent genes in naïve mESCs with a (d) 10% or (e) 50% HMD threshold. (f) Heatmaps and metaprofiles of bivalent genes from naïve mESCs. (g) Metaprofiles of H3K4me3, H3K27me3, and bivalency at genes tracked from primed mESCs to NPCs for bivalent genes in primed mESCs (>25% HMD). (h) Heatmaps and metaprofiles of bivalent genes in primed mESCs that are not bivalent in naïve mESCs.

Extended Data Fig. 4 | Comparing our bivalent genes to other studies. (a-b) Contingency tables and metaprofiles for genes that are identified as >25% bivalent in our study and by Mikkelsen et al.¹⁸, Mas et al.³⁵, and Xiao et al.⁵⁸, wherein: (a) gene is identified as bivalent in the external study if overlapping H3K4me3 and H3K27me3 peaks overlap the 0 to +400bp region of a gene relative to the TSS, or (b) gene is identified as bivalent in the external study if overlapping H3K4me3 and H3K27me3 peaks overlap the region from 2.5kb upstream of the TSS to the end of the gene²². (c) Overlap of bivalent genes from external datasets (as defined in part a) with each of our bivalent gene dominance classes in naïve mESCs. Significance computed by two-tailed Fisher hypergeometric test. (d) Overlap of genes with bivalency HMD > 25% and with H3K4me3 + H3K27me3 HMD > 25% in all three cell states.

Extended Data Fig. 5 | Bivalency changes across differentiation by modification dominance

class. (a-c) Metaprofiles of H3K4me3, H3K27me3, and bivalency for bivalent genes (>25% HMD) in naïve mESCs that are (a) H3K27me3 dominant ($H3K27me3/H3K4me3 > e^{-1}$), (b) H3K4me3 dominant ($H3K27me3/H3K4me3 < e^{-1}$), or (c) have no dominance in naïve mESCs, tracked through three cell states. **(d-e)** Metaprofiles of H3K4me3, H3K27me3, and bivalency for bivalent genes (>25% HMD) in primed mESCs that are for indicated dominance classes tracked from primed mESCs to NPCs.

Extended Data Fig. 6 | Methyltransferase assays identifying potential pathways for

establishment of bivalency. (a-b) Methyltransferase assays for MLL1, MLL2, MLL3, MLL4, and Set1B core HMTase complexes using (a) 15 ng/uL (n=6) and (b) 20 ng/uL (n=5) semisynthetic nucleosomes as substrates for methylation. Endpoints were established at 180 min by kinetic evaluation to be sensitive to difference in activity for this panel. Signal is corrected for background and no nucleosome substrate activity. Error bars represent standard deviation.

Extended Data Fig. 7 | HMTase peaks and bivalency. Contingency tables and metagene

profiles in naïve mESCs for genes with and without overlapping HMT peaks. Ezh1 and Ezh2 peaks were identified as Suz12 peaks lost upon Ezh1 or Ezh2 knockout⁸². Set1A peaks were identified by ChIP against Set1A⁶². Mll2 peaks were identified by ChIP against Mll2⁴⁵.

Extended Data Fig. 8 | Bivalency and differential gene expression. (a-b) Violin plots of gene

expression for (a) all genes and (b) bivalent (>25% HMD) genes in each cell state. **(c)** Bivalency

metaprofiles in naïve mESCs at promoters binned by gene expression decile. **(d)** Violin plots of bivalency HMD in naïve mESCs at promoters with and without CpG islands. Inset shows proportion of genes that are bivalent in sets of genes classified by CpG content: high-CpG promoters (HCP), intermediate-CpG promoters (ICP), and low-CpG promoters (LCP), defined as previously described by Mikkelsen et al.¹⁸. Total number of genes in each class is provided as n. **(e)** Violin plots of DNA methylation at bivalent and non-bivalent genes (top), broken by dominance class for bivalent genes (bottom). **(f-g)** Violin plots of gene expression in (f) non-bivalent (<25% HMD) and (g) bivalent (>25% HMD) genes from naïve mESCs that are H3K27me3 dominant ($H3K27me3/H3K4me3 > e^{-1}$; left), have no clear dominance (centre), or are H3K4me3 dominant ($H3K27me3/H3K4me3 < e^{-1}$; right). **(h-k)** Metaprofiles of H3K4me3, H3K27me3, and bivalency at genes tracked from naïve mESCs to primed mESCs to NPCs for (h) DEGs, (i) non-DEGs, (j) genes upregulated from naïve mESCs to NPCs, and (k) genes downregulated from naïve mESCs to NPCs. **** $p < 10^{-16}$ (Welch's two-tailed t-test).

Extended Data Fig. 9 | Bivalency at different classes of genes. **(a)** Metaprofiles of H3K4me3, H3K27me3, and bivalency at genes tracked from naïve mESCs to primed mESCs to NPCs for bivalent genes of indicated gene ontology terms. **(b)** Metaprofiles of H3K4me3, H3K27me3, and bivalency at genes tracked across differentiation for genes that lose bivalency at the promoters (0 to +400bp relative to TSS) from naïve mESCs (>25% HMD) to NPCs (<10% HMD) and are upregulated (top) or downregulated (bottom) over differentiation.

Extended Data Fig. 10 | Modelling the additional information content provided by bivalency over H3K4me3 and H3K27me3 alone. **(a)** ROC curves for identifying DEGs from

primed mESCs to NPCs by H3K4me3, H3K9me3, H3K27me3, bivalency, $\ln(\text{H3K27me3}/\text{H3K4me3})$, or DNA methylation in primed mESCs. For each point, parameter value threshold used to compute true positive rate (TPR) and false positive rate (FPR) is indicated by the colour. Traits with thresholds identifying non-DEGs rather than DEGs are marked with “rev.” **(b)** Bayes Information Criterion (BIC) for logistic models identifying DEGs from naïve mESCs or primed mESCs to NPCs with different parameters. **(c)** Legend for generalized linear models (GLMs). **(d)** Accuracy of trivial model and GLMs by threshold accuracy (gene identified as DEG if logistic regression > 0.5 ; left) and by ROC area under curve (right). **(e)** ROC curves for identifying DEGs from primed mESCs to NPCs by different GLMs. For each point, logistic regression threshold value used to compute TPR and FPR is indicated by the colour.

Supplementary Notes

Supplementary Note 1

As each nucleosome has two H3 protomers, there are several different configurations of bivalency that a bivalent nucleosome can theoretically adopt, each with a different avidity for ChIP pulldown with immobilized antibody. At one extreme, with the highest avidity, is the symmetric *cis*-bivalency form, where both H3K4 and both H3K27 residues are trimethylated (Extended Data Fig. 1e). This nucleosome has the most epitopes for antibody binding and will thus have the highest avidity in pulldown reflected in apical pulldown efficiency (Extended Data Fig. 1d). At the other extreme, with the lowest avidity, is the *trans*-bivalency form, where single H3K4me3 and H3K27me3 marks decorate different histone tails (Extended Data Fig. 1e). This has the fewest epitopes for antibody binding and will thus have no avidity in pulldown.

This poses a theoretical challenge in normalization and calibration of a ChIP study; because we cannot separately measure *trans*-bivalency, symmetric *cis*-bivalency, nor any intermediate states, it is impossible for us to definitively state whether a given locus with a given HMD has relatively few nucleosomes that are symmetric *cis*-bivalent or whether it has relatively many nucleosomes that are *trans*-bivalently modified. To accommodate for this limitation, we include two different bivalent calibrants in our set of nucleosome standards: one that is symmetric *cis*-bivalent and one that is *trans*-bivalent. The bivalency sequential ChIP can then be normalized to either one of these standards, and because these two cases represent the limits of pulldown avidity, normalization to these calibrants will define the theoretical “range” in which true bivalency HMD (i.e. the proportion of nucleosomes with some bivalent configuration) exists (Extended Data Fig. 1e). We note that, because the signal from calibration to these standards are scalar multiples of each other, we cannot uniquely distinguish these two configurations in the

genome. Absent any prior information about the dominant configuration of bivalency, the proportion of bivalently modified nucleosomes at a given locus will exist in the range defined by calibration to symmetric *cis*- or *trans*-bivalent standards (Extended Data Fig. 2a).

In practice, there are a few reasons why this is not a major concern. First, there is no mass spectrometry evidence that H3K4me3 and H3K27me3 exist on the same histone tail, despite specific enrichment for these marks and sensitive detection limits^{49,81}, suggesting that configurations other than *trans*-bivalency are at most, extremely minor in abundance. Second, the scarcity of these *cis*-tail modifications is consistent with the biochemical literature prior to this work that suggests the biogenesis of these *cis*-tail modifications is enzymatically challenging due to antagonistic allosteric effects (see Supplementary Note 4). Third, even if symmetric *cis*-bivalency does exist at some loci, for the purposes of tracking changes in bivalency across differentiation, we can still observe an increase or decrease in bivalency by this calibration method; we simply cannot precisely discern whether the effect is driven by nucleosomes gaining/losing *trans*-bivalency, *cis*-bivalency, or some combination of the two. The overall amount of bivalency would still increase or decrease in all those scenarios, and so long as our choice of calibrant remains consistent, we can still measure that change regardless of the calibrant that we use for our normalization. Therefore, though we have generated datasets using both calibrants, we present analyses of our reICeChIP bivalency pulldowns calibrated to the *trans*-bivalent standards.

Supplementary Note 2

Throughout this study, we have defined gene promoters to be the region from 0 to +400bp relative to the TSS, representing the +1 and +2 nucleosomes of each gene. These nucleosomes

tend to be well-positioned⁸³ and, accordingly, are most likely to provide us with adequate read depth to robustly quantify each histone modification. This definition is conservative; we find that H3K4me3 and bivalent domains, which tend to be peak-like, have a median breadth of 550bp at bivalent genes (Extended Data Fig. 2d).

The width of these domains raises an important point regarding the measurement of histone modification density as a continuous variable. At a given nucleosome in a single allele of a single cell, there are only three possible states for a histone modification: symmetric, asymmetric or not present. However, nucleosome readers do not typically bind only a single nucleosome at a single position; rather, the local density of the modification across multiple nucleosomes is crucial in localizing these effectors through multivalent avidity-based interactions^{84–87}. Indeed, we find that the HMD across sequential nucleosomes relative to the TSS is well autocorrelated (Extended Data Fig. 2e). This means that the interpretation of the HMD across a multinucleosomal span becomes more nuanced; a given histone modification may exist at one or more of those nucleosomes. Accordingly, despite the fact that a single nucleosome is essentially ternary in whether it has a given histone modification or not (i.e. HMD of 0% or 100%), a region spanning multiple nucleosomes could have an intermediate HMD; it is this latter quantity that is most relevant for the biological function imparted to the nearby genomic regions, and this is the quantity we analyse through this work.

Supplementary Note 3

For the datasets presented in this work, the vast majority of promoters have a histone modification density between 0-100%, representing the proportion of nucleosomes at those

promoters with the modification of interest (Fig. 2c; Extended Data Fig. 3a). However, at some loci, the measured HMD exceeds 100%. There are several possible reasons for this.

The most important of these possibilities is low input depth. The ICeChIP datasets are normalized to the input read depth at every genomic interval to accommodate for differences in local nucleosome density when computing the HMD. However, this means that at regions that are relatively nucleosome-depleted, there will be few reads in the input, meaning that the denominator of the HMD computation is quite small (Methods). This increased Poisson noise in these regions of low input can result in inflated apparent HMD beyond the physical limit of 100%. To accommodate for this, we can compute 95% confidence intervals for the HMD of each modification at each genomic position, and these confidence intervals virtually always overlap the physically possible range of HMD values (e.g., Fig. 1c). In naïve mESCs, only 0.5% of the promoters have a bivalency HMD above 100%, and for the vast majority of these promoters (86.1%), the 95% confidence interval error estimate ranges below 100%. The fact the apparent bivalency HMD calibrated by trans-bivalent standards, is broadly constrained to less than 100% further supports the idea that this choice of calibrant is appropriate and not inflationary (Supplementary Note 1).

There are also several other possibilities that are more challenging to accommodate for. First, some regions of the genome are known to be more artefact-prone for sequencing and mapping⁸⁸; if the IP sample is enriched for these sequences relative to the input, then that could be disproportionately represented in the IP and have an apparent HMD greater than 100%. Second, the antibodies themselves could skew the apparent HMD. If the antibody is capturing substantial off-target material, then that will result in systematic inflation of the IP, resulting in an inflated HMD. Though ICeChIP barcoded nucleosome standards can help monitor off-target

pulldown of some nucleosome species, we can only measure the capture of the standards that we actually have spiked into the experiment. If we do not have nucleosome standards available for a potential off-target modification, then we cannot definitively state that the antibody is not capturing that material. In this context, that is likely most important for H3K27me3 pulldowns; though we cannot state this definitively due to the lack of H3K27me2 standards, it is plausible that we are pulling down some amount of H3K27me2 with these IPs, resulting in slightly inflated apparent H3K27me3 HMD. However, this may not be too problematic; H3K27me2 and H3K27me3 are thought to be recognized by many of the same proteins and to have highly similar functions⁸², so the conflation of the two – if present – likely does not pose a significant problem in ascribing biologic function.

On a related note, at some loci, the bivalency HMD goes below 0%. In naïve mESCs, 8.8% of the promoters have a bivalency HMD below 0%, yet for the vast majority of these promoters (90.8%), the 95% confidence interval error estimate ranges above zero. This is because we employ *in silico* signal-correction for the bivalency dataset to remove signal that is attributable to H3K9me3. In essence, we can measure the amount of H3K9me3 pulldown in our bivalency ICeChIP dataset due to nucleosome standards employed, and we can separately measure the H3K9me3 HMD by a highly specific IP. We can then a linear combination correction matrix to remove the signal that is attributable to directly measured H3K9me3 at these loci. This method can effectively reduce the impact of modest off-target binding H3K9me3, but at some loci, will result in a subzero apparent HMD due to random sampling of read depth in the two distinct pulldowns employed.

Finally, at some sets of gene promoters, the trans-bivalency HMD is shown to be greater than the H3K4me3 or H3K27me3 HMD. This apparent discrepancy has a few possible reasons.

First, there is some nuance in the interpretation of HMD in the context of single-target ICeChIP and reICeChIP. A nucleosome has two copies of each of its core histone proteins, including histone H3. This means that there are two possible sites of modification on each nucleosome for each individual modification; if only one of those sites is modified, then that corresponds to an HMD of 50% because only half the possible modification sites are actually modified. However, this is different for the trans-bivalency HMD; by definition, only one trans-bivalency modification pattern can exist on a given nucleosome at any given time. If two “trans-bivalent” modification patterns existed on the same nucleosome simultaneously, then both H3K4 and both H3K27 residues would be trimethylated – which is symmetric *cis*-bivalency. As such, if one H3K4 and one H3K27 residue are trimethylated, then 100% of the possible trans-bivalency configurations for the nucleosome of interest are satisfied, meaning that the trans-bivalency HMD will be 100%. However, in this case, the H3K4me3 and H3K27me3 HMDs will only be 50% because only half the modifiable residues are actually modified.

The other caveat is that symmetrically modified nucleosomes will be pulled down more efficiently than asymmetrically modified nucleosomes due to avidity effects, as can be seen in the pulldown of symmetric vs. asymmetric H3K4me3 and *cis*-bivalency vs. *trans*-bivalency (Extended Data Fig. 2), and observed previously⁴⁶. This means that calibration to symmetric nucleosome standards will have a larger denominator in computation of HMD and thereby yield lower apparent HMDs; this can also contribute to the lower apparent HMD of H3K4me3 and H3K27me3 relative to trans-bivalency. Accommodating for this phenomenon would require detailed profiling of asymmetric H3K4me3 (which is currently difficult due to the low quality of H3K4me0 antibodies), asymmetric H3K27me3 (which is not currently possible), and distinguishing between trans-bivalency and cis-bivalency (which is also not currently possible).

However, as noted in Supplementary Note 1, so long as the method of calibration remains consistent, increases in apparent HMD will still correspond to increases in the modification of interest. Whether that increase in the target modification is due to asymmetric modification becoming symmetric or due to new gain of the modification at a previously unmodified locus in an instantaneous subpopulation remains unclear, but in both cases, modification density is still being gained at that locus. As such, even with these caveats, we can still quantitatively compare different datasets to each other as we use consistent calibration standards.

Supplementary Note 4

Intriguingly, the catalytic activity of the EZH2-PRC2 core complex on nucleosome substrates is potentiated by pre-existing H3K27me3^{89,90}, yet inhibited by H3K4me3, particularly when symmetric^{49,56,59}. Conversely, symmetric H3K27me3 has been reported to modestly inhibit several of the human COMPASS-family complexes by qualitative assays, although only SET1 complexes were examined at the nucleosome level⁶⁰. This presents a potential concern for our data – if the enzyme complexes that install these marks are mutually antagonized by the opposing mark, how might the widespread bivalency we observe arise? As the PRC2 effects are well established with detailed quantitative enzymology^{49,56,59}, which we recapitulate (data not shown), we deployed more quantitative HMTase assays with a larger panel of relevant nucleosomal substrates to evaluate the COMPASS/SET1B/MLL-family core complexes for allosteric modulation by preexisting marks (Extended Data Fig. 6).

Supplementary Note 5

In this context, sensitivity refers to the proportion of DEGs that are represented in a specific class of genes (e.g. H3K27me3-dominant bivalent genes), whereas specificity refers to the proportion of that class of genes that are differentially expressed. Under the prevailing bivalency model, bivalency is associated with poised genes that become upregulated or downregulated upon differentiation; as such, it should have high specificity for DEGs.

Supplementary Note 6

The first way we evaluate different models for predicting DEGs is to compute the Bayes Information Criterion (BIC). Though not definitive, this metric estimates whether addition of a parameter to a model improves it more than would be expected from chance alone. When comparing two models, the model with the lower BIC will tend to have more explanatory parameters and/or fewer non-explanatory parameters than the model with the higher BIC. To this end, if BIC increases when a parameter is added, then it can be interpreted that the parameter being added contributes minimal additional explanatory power. Here, we find that adding bivalency to a model increases the BIC, meaning that it is likely (though not definitively) not contributing meaningfully more information in predicting DEG status in this differentiation paradigm.

A more definitive way to evaluate model accuracy is to use hold-out cross-validation. In this method, we split the set of all genes into two groups, one with 80% of the genes (the training set) and one with 20% of the genes (the testing set). We then train our GLMs on the training set and use the derived models to predict DEG status in the testing set. Hold-out cross-validation is a highly effective way of testing whether a model is overfit or underfit upon addition or removal of

a parameter. If model accuracy increases substantially, then that would suggest the parameter has explanatory power over that provided by the other parameters. Conversely, if model accuracy decreases substantially, then that suggests that the additional parameter causes overfitting. Minimal changes in model accuracy suggest that the additional parameter contributes little to the model over the existing parameters, positively or negatively.

There are two metrics we use to test the accuracy of the predictions in the testing set. The first is by logistic regression thresholding, in which the gene is predicted to be a DEG if the modelled probability is greater than 0.5. The second is by computing the area under the receiver operator characteristic curve to measure true and false positive rates using different modelled probabilities as the thresholds.

Overall, we find that the GLM with bivalency barely changes model accuracy by either metric on hold-out cross-validation, with the magnitude of change being similar to that observed by instead adding H3K9me3 or DNA methylation. As such, we can interpret that none of these parameters – including bivalency – meaningfully contributes to the prediction of DEGs beyond what can be achieved with H3K4me3 and H3K27me3 in this system.

Methods

Cell Culture

Naïve mouse Embryonic Stem Cells (mESCs) were grown from the mESC E14 cell line (129/Ola background)⁹¹ in high glucose DMEM (Invitrogen), supplemented with 15%(v/v) FBS (Gibco), 1%(v/v) non-essential amino acids (Gibco), 1x penicillin/streptomycin (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 2mM L-glutamine (Gibco), 1000 U/mL LIF (ESG1107 Millipore), 3 µM CHIR99021 (LC Laboratories), 1 µM PD0325901 (LC Laboratories), sterilized using 0.1 µm filter flask (Millipore), stored up to 1 week in 4°C.

Primed mESCs were grown from the mESC E14 cell line (129/Ola background)⁹¹ in high glucose DMEM (Invitrogen), supplemented with 15%(v/v) FBS (Gibco), 1%(v/v) non-essential amino acids (Gibco), 1x penicillin/streptomycin (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 2mM L-glutamine (Gibco), 1000 U/mL LIF (ESG1107 Millipore), sterilized using 0.1 µm filter flask (Millipore), stored up to 1 week in 4°C.

Naïve and primed mESCs were grown on plates coated with 0.1% bovine gelatin (Sigma), grown to 70-90% confluence and passaged daily at a 1:3 ratio, with a media change 3 hours before passaging, supplemented with 1 vol. of fresh media 8 hours after passaging.

To initiate the adherent monolayer differentiation process to neuronal progenitor cells (NPCs; Day 0)^{92,93}, naïve mESCs cells were split onto a gelatinized 6 cm plate at 1 x 10⁴ cells/cm² and allowed to grow for 24 hours. On Day 1, the media was switched to RHB-A (Takara, Y40001) and was subsequently changed every other day. On day 4, cells were split and plated onto Poly-L-Ornithine, laminin-treated 6-cm plates. Prior to cell seeding the plates were treated with 0.01% Poly-L-Ornithine (Millipore, A004C) for at least 20 min, followed by 5 ug/cm² of laminin (Fisher, CB40232) resuspended in basal RHB-A medium (Takara, Y40000). After washing off

this treatment, cells were seeded in fresh RHB-A, supplemented with 10 ng/mL of bFGF (PeproTech, 100-18B) and EGF (PeproTech, 315-09). Cells were then split every 4 days at $\geq$ 20,000 cells/cm² until an appropriate amount of NPCs were cultured for ICeChIP.

Semi-synthetic Histone Preparation

Human histones H3.2(C110A)K4me₃, H3.2(C110A)K9me₃, H3.2(C110A)K27me₃, H3.2(C110A)K4me₃-K27me₃ were made by semi-synthesis as described previously^{46,94}. Asymmetric disulfide linked histone H3K4me₃ - H3K27me₃ dimers were made by semi-synthesis as described previously⁵⁶.

Octamer Reconstitution

Symmetrical H3K4me₃, H3K27me₃, H3K4me₃-K27me₃ and H3K9me₃ octamers were made as previously described^{95,96}. Briefly, equimolar amounts of histone H2A, H2B, H3 and H4 were mixed to the final concentration of 1 mg/ml in unfolding buffer (50 mM Tris-HCl pH 8, 6.3 M Guanidine-HCl, 10 mM 2-mercaptoethanol, 4 mM EDTA), subsequently they were loaded into 3500 M.W.C.O. dialysis tubing (Pierce Snakeskin) and dialyzed in 1000 volumes of refolding buffer (20 mM Tris-HCl pH 7.5, 2 M NaCl, 1mM EDTA, 5 mM DTT), overnight at 4°C. Dialyzed sample was 0.22 μ m filtered, and octamers were resolved by S200 gel filtration chromatography (Superdex 200 10/300 GL, GE Healthcare) using refolding buffer as mobile phase. Eluted octamer fractions were pooled and concentrated using centrifugal filters (Amicon Ultra-4, 10k M.W.C.O., Millipore) to a final concentration of 5-15 μ M, diluted with 1 volume of octamer storage buffer (20 mM Tris-HCl pH 7.5, 2 M NaCl, 1 mM EDTA, 5 mM DTT, 55% glycerol), and stored in -20°C. Concentration of octamer was measured spectroscopically using

concentrator flow-through as a blank, $\epsilon_{280\text{nm}}=44700 \text{ M}^{-1}\text{cm}^{-1}$, $M_{\text{oct}}\approx 108500 \text{ g}^1\text{mol}^{-1}$. Octamers were visualized using 18% separating (4% stacking) discontinuous Laemmli SDS-PAGE in Mini-Protean gel running system (Bio-Rad) run for 70 minutes at 22mA, 200V max.

Asymmetrical H3K4me3, H3K27me3 octamers were done as above with the following differences. Equimolar amounts of histone H2A, H2B, H3 and H4 were mixed in unfolding buffer to the total of 1-2 mg, where 90% of histone H3 was trimethylated on Lys 4 or Lys 27 and remaining 10% were unmethylated and had His₆-tag at N-terminus with TEV cleavage site. Octamers were reconstituted overnight by dialysis in 1000 volumes of phosphate refolding buffer (50 mM Na_xPO₄ pH 7.5, 2M NaCl), at 4°C. Octamers were purified by S200 gel filtration chromatography, and his-tagged octamers were isolated using cobalt-based immobilized metal affinity chromatography Dynabeads magnetic particles. Octamers were incubated with magnetic beads for 10 min at 4°C on rotator, followed by two 1 ml washes (50 mM Na_xPO₄ pH 7.5, 2 M NaCl, 10 mM imidazole), and eluted with 50 ul of elution buffer (50 mM Na_xPO₄ pH 7.5, 2 M NaCl, 250 mM imidazole, 1 mM EDTA, 1 mM DTT), the elution step was repeated 6 times, fractions were characterized spectroscopically, pooled, diluted with 1 volume of octamer storage buffer, and stored in -20°C.

Asymmetrical *trans*-bivalent H3K4me3-K27me3 octamers were prepared the same way as symmetrical octamers with the following differences. Histones H2A, H2B, H4 and asymmetric disulfide linked histones H3K4me3 - H3K27me3 were mixed 1.2 : 1.2 : 1 : 0.5 ratio. Remaining steps were done as previously described but no reducing agents were used until octamer particles were formed.

All other octamers were obtained from EpiCypher, Inc.

Nucleosome reconstitution

DNA barcodes were constructed based on 601 nucleosome positioning sequence⁹⁷. One or both ends of 601 Widom sequence were substituted with 24 bp “barcode” sequence. Each barcode sequence is comprised of two 11 bp sequences absent in human and mouse genome, and constant 2bp linker DNA is added on a free end of the 601 nucleosome positioning sequence.

Nucleosomes were reconstituted as previously described⁴⁶. Briefly, 10-100 pmol DNA and histone octamers were mixed in 1 : 1 ratio, at a final concentration >1 μ M, and dialyzed in dialysis buffers (Hampton Research) against a non-linear gradient of sodium chloride 2M NaCl $\rightarrow$ 0.2M NaCl in a buffer containing 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol over the course of 12-16 hours⁸⁴. Afterwards nucleosomes were recovered, diluted with 1 volume of 2x storage buffer (20 mM Na•Cacodylate pH 7.5, 10% v/v glycerol, 1 mM EDTA, 1x RL Protease Inhibitor Cocktail [1 mM PMSF, 1 mM ABESF, 0.8 μ M aprotinin, 20 μ M leupeptin, 15 μ M pepstatin A, 40 μ M bestatin, 15 μ M E-64]), and stored at -20°C. Nucleosome concentration was measured by densitometry of 2% agarose gels, 1x TBE (89 mM tris-base, 89 mM boric acid, 2 mM EDTA) run for 30 minutes in 5V/cm electrical field gradient, followed by staining with 1x SYBR Gold (Invitrogen) for >30 minutes. Prior to electrophoresis, nucleosomes were disassembled with 2 M NaCl, roughly 1 pmol of nucleosomes were loaded per well and measured in triplicate against known quantity of free DNA of the same size. For use as ICeChIP standards, the semi-synthetic nucleosomes were diluted to 1 nM concentration using long-term storage buffer (10 mM Na•Cacodylate pH 7.5, 100 mM NaCl, 50% Glycerol, 1 mM EDTA, 1x RL Protease Inhibitor Cocktail, 100 μ g/mL BSA(NEB)) and stored at -20°C.

ICeChIP – input preparation

ICeChIP has been done as previously described^{46,54}. Briefly, 10^7 - 10^8 plate adherent cells were released using Accutase (Millipore), quenched with complete medium and collected (500 rcf, 5 min., 4°C). Subsequent steps have been done on ice, cells after each wash were collected by centrifugation (500 rcf, 5 min., 4°C). Cells were washed twice with 10 ml PBS, twice with 5ml buffer N (15 mM Tris pH 7.5, 15 mM NaCl, 60 mM KCl, 8.5% (w/v) Sucrose, 5 mM MgCl₂, 1 mM CaCl₂ 1 mM DTT, 1x RL Protease Inhibitor Cocktail). Cells' membranes was lysed by adding 1 volume of the 2x Lysis Buffer (Buffer N supplemented with 0.6% NP-40 substitute (Sigma)) to the single cell suspension resuspended in 2 PCVs (packed cell volumes) of Buffer N. After 10 minutes incubation on ice, nuclei were collected by centrifugation and resuspended in at least 6 PNV (packed nuclei volumes) of buffer N. Subsequently, nuclei were layered over 7.5 ml Sucrose Cushion N (15 mM Tris pH 7.5, 15 mM NaCl, 60 mM KCl, 30% (w/v) Sucrose, 5 mM MgCl₂, 1 mM CaCl₂ 1 mM DTT, 1x RL Protease Inhibitor Cocktail, 50 µg/mL BSA(NEB)) in a 50 ml centrifuge tube. Nuclei were spun through the sucrose cushion in swinging bucket rotor at 500 rcf for 12 min., 4°C. Nuclei were resuspended in 2 PNVs of buffer N, total nuclei acid content was measured spectroscopically at 260 nm by Nanodrop (Thermo Scientific) ($1A_{260} = 50 \text{ ng}/\mu\text{l}$), prior to measurement, DNA was stripped from chromatin by adding 18 µl – 98 µl of 2 M NaCl and DNA was fragmented by vortexing and water bath sonication. The quality and quantity of nuclei was measured using a hemocytometer. The apparent concentration of chromatin was adjusted to 1 µg/µl with Buffer N. The following semi-synthetic standards were then spiked in: symmetrical H3K4me3, H3K9me3, H3K27me3, H3K36me3, H3K79me2, H3K4me3-K27me3 *cis*-bivalent, asymmetrical H3K4me3, H3K4me3-K27me3 *trans*-bivalent. To fragment the DNA, we aliquoted 100 µg of chromatin and added 1 Worthington unit of

Micrococcal nuclease (Worthington) per 4.785 µg of chromatin (measured at 260nm, $1A_{260} = 50 \text{ ng}/\mu\text{l}$) and incubated at 37°C for 12 minutes. Digestion was stopped by adding 1/10 volume of 11x MNase stop buffer (110 mM EGTA, 110 mM EDTA pH 8.0). Subsequently, nuclei were lysed by slowly adding 5 M NaCl while mixing on a vortex (lowest setting) to the final concentration of 600 mM NaCl. Insoluble material has been spun down at 18000 rcf, 1min., 4°C.

Hydroxyapatite (HAP) chromatography has been done as described previously⁴⁶. Briefly, 66 mg of HAP resin (Bio-Rad Macro-Prep® Ceramic Hydroxyapatite Type I 20 µm) was rehydrated with 200 µl of HAP buffer 1 (3.42 mM Na₂HPO₄ and 1.58 mM NaH₂PO₄ final pH 7.2, 600 mM NaCl, 1 mM EDTA, 200 µM PMSF). Subsequently, 100 µg of digested soluble chromatin was added to the rehydrated resin and incubated for 10 minutes at 4°C on a rotator. Afterwards, resin slurry was transferred to the centrifugal filter unit (Millipore Ultrafree MC–HV Centrifugal Filter 0.45 µm). Resin was washed 4 times with 200 µl of HAP buffer 1, 4 times with 200 µl of HAP buffer 2 (3.42 mM Na₂HPO₄ and 1.58 mM NaH₂PO₄ final pH 7.2, 100 mM NaCl, 1 mM EDTA, 200 µM PMSF), and eluted 3 times with 50 µl HAP elution buffer (342 mM Na₂HPO₄ and 158 mM NaH₂PO₄ final pH 7.2, 100 mM NaCl, 1 mM EDTA, 200 µM PMSF), each wash/elution step was accompanied by centrifugation step (600 rcf, 30 sec., 4°C). Concentration of the chromatin was evaluated spectroscopically by Nanodrop (Thermo Scientific) ($1A_{260} = 50 \text{ ng}/\mu\text{l}$). Apparent concentration of the chromatin was adjusted to 20 ng/µl with ChIP buffer 1 with BSA (25 mM Tris pH 7.5, 5 mM MgCl₂, 100 mM KCl, 10% (v/v) glycerol, 0.1% (v/v) NP-40 substitute, 100 µg/ml BSA(NEB)).

ICeChIP – immunoprecipitation

771 ICeChIP was performed as previously described⁴⁶ with following modifications: α -H3K4me3
772 ChIP- 5 μ g chromatin, 2 μ g 304M3B-1xHRV3C⁵³, 40 μ l Streptavidin M-280 Dynabeads
773 (Invitrogen). α -H3K9me3 ChIP- 3 μ g chromatin, 0.5 μ g 309M3B⁵³, 10 μ l Streptavidin M-280
774 Dynabeads (Invitrogen). α -H3K27me3 ChIP - 0.8 μ g chromatin, 0.6 μ g CST C36B11 lot 8, 5 μ l
775 Protein G Dynabeads (Invitrogen). Aforementioned volumes of magnetic beads were washed
776 twice with 200 μ l ChIP buffer 1 with BSA. Antibodies were resuspended in 100 μ l of ChIP
777 buffer 1 with BSA; subsequently, magnetic beads were collected using magnetic rack,
778 supernatant was removed, and magnetic beads were resuspended with the antibody solution and
779 incubated for at least 1 hr, 4°C, on a rotator. Afterwards unbound antibody was washed away
780 with two 200 μ l washes of ChIP buffer 1 with BSA. Streptavidin beads were additionally washed
781 twice with 200 μ l of ChIP buffer 1 with BSA supplemented with 5 μ M biotin for 10 min, at 4°C,
782 on a rotator for each wash, followed by wash with 200 μ l ChIP buffer 1 with BSA. Supernatant
783 was removed on a magnetic rack and specific amount of chromatin, mentioned at the beginning
784 of this chapter, was used to resuspend the magnetic beads. Chromatin was incubated with
785 antibody-beads conjugates for 10-15 minutes, at 4°C, on a rotator.

786 Subsequently, magnetic beads were washed two times with 200 μ l ChIP buffer 2 (25 mM Tris
787 pH 7.5, 5 mM MgCl₂, 300 mM KCl, 10% (v/v) glycerol, 0.1% (v/v) NP-40 substitute, 100 μ g/ml
788 BSA(NEB)), and one time with 200 μ l ChIP buffer 3 (10 mM Tris pH 7.5, 250 mM LiCl, 1 mM
789 EDTA, 0.5% Na•Deoxycholate, 0.5%(v/v) NP-40 substitute, 100 μ g/ml BSA(NEB)), 10
790 minutes, at 4°C, on a rotator with tube change after each wash. These washes were followed by
791 quick 200 μ l ChIP buffer 1 (without BSA) wash, and 200 μ l TE wash (10 mM Tris-HCl pH 8.0,
792 1 mM EDTA). Chromatin was released from the resin using 50 μ l ChIP elution buffer (50 mM
793 Tris pH 7.5, 1 mM EDTA, 1% (w/v) SDS) at 55°C, for 5 minutes. Elution was supplemented

with 200 mM NaCl, 10 mM EDTA, 10 µg of Proteinase K (Roche) and incubated at 55°C for 2 hours. DNA was isolated using 3 volumes of Serapure HD (1 mg/ml of 1 µm, hydrophobic, carboxylated, Sera-Mag SpeedBeads (GE 65152105050250), 20% PEG-8000, 2.5 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.05% Tween-20, filter sterilized prior to addition of magnetic beads), incubated for 5 minutes at room temperature, collected with magnetic rack and washed twice with >200 µl of 75% ethanol, on a magnetic rack without disturbing the magnetic beads. Subsequently, ethanol was carefully removed, and DNA was eluted by resuspending beads in 50 µl of TE buffer, magnetic beads were collected using magnetic rack and supernatant was moved to a new tube. In order to limit DNA loss, all operations have been performed using 250 µl siliconized tubes.

reICeChIP

reICeChIP was performed in the same manner as described above with following changes. For the primary IP we have used 5 µg of HAP purified chromatin, 2 µg 304M3B-1xHRV3C – HRV 3C cleavable, biotinylated, αH3K4me3 Fab (PDB:4YHZ)⁵³, immobilized on 40 µl Streptavidin M-280 Dynabeads (Invitrogen). After 10 minutes incubation of chromatin with antibody-resin conjugate, resin was washed three times with 200 µl ChIP buffer 1 with 100 µg/ml BSA, each wash consisted of 10 minutes incubation at 4°C, on a rotator, followed by tube change. Subsequently, resin was briefly washed with 200 µl ChIP buffer 1 with 100µg/ml BSA, and chromatin was released from the resin with 20 µl of ChIP buffer 1 supplemented with 100 µg/ml BSA and 4 µg HRV3C incubated (GE Healthcare) on ice for 60 minutes, elution step was repeated one more time and both elutions were combined. HRV 3C endoprotease efficiently cleaves at its target sites at 4°C⁵⁵ and has a wide range of chemical tolerance⁹⁸, but it highly-

specific for its cognate cleavage sequence⁹⁹ permitting facile use in ChIP under conditions which preserve nucleosomes. Primary elution was added to 0.6 µg CST C36B11, αH3K27me3 mAb, immobilized on 5 µl of Protein G Dynabeads(Invitrogen) for 10 minutes, at 4°C, on a rotator. Subsequently, magnetic beads were washed two times with 200 µl ChIP buffer 2, and one time with 200 µl ChIP buffer 3, 10 minutes, at 4°C, on a rotator, with tube change after each wash. These washes were followed by quick wash with 200 µl ChIP buffer 1 (without BSA). Chromatin was released from the resin by 5 minutes incubation with 50 µl ChIP elution buffer, at 55°C. Chromatin was Proteinase K digested, and DNA was purified as described in ICeChIP – immunoprecipitation chapter.

Design, expression, and purification of 304M3B-1xHRV3C

304M3B-1xHRV3C Fab is based on previously described Fab 304M3B(PDB:4YHZ)⁵³. The gene encoding the Fab was modified to contain HRV3C cleavage site at the C-terminus of the heavy chain. To that end, we inserted SSSLEVLFFQGP (AGC AGC AGC CTT GAA GTC CTC TTT CAG GGA CCC) sequence just after the position T229 of heavy chain (numbered as in PDB:4YHZ) and before biotinylation acceptor peptide (GLNDIFEAKIEWHE)¹⁰⁰. The Fab was expressed in the 55244 strain of *E.coli* in the TBG media (Terrific Broth (FisherBrand), 0.8% (v/v) glycerol) with 100 µg/ml carbenicillin, grown for 24 hours, at 30°C, 200 rpm in the Fernbach non-baffled flasks, with constricted airflow. Fab was purified using Protein G-A1¹⁰¹ affinity chromatography, followed by cation-exchange chromatography (Resource S, GE Healthcare). Purified Fab was *in vitro* biotinylated using BirA biotin ligase.

Sequencing and Data Analysis

Each sequencing library was made using 10 ng of DNA. Illumina sequencing libraries were made with NEBNext Ultra II DNA Library Prep for Illumina (NEB), according to the manufacturer protocol. DNA libraries were amplified using 8 PCR amplification cycles (C1000, Bio-Rad). Cluster generation and sequencing was performed using the standard Illumina protocols for Illumina HiSeq 4000 by the University of Chicago Functional Genomics Core facility. Data analysis was performed as previously described⁴⁶. Briefly, reference genome was modified to contain sequences of semi-synthetic nucleosome barcodes. Reads were mapped to GRCm38/mm10+barcodes reference genome, using Bowtie2¹⁰², end-to-end, sensitive preset. Subsequently, SAM files were filtered to reject unmapped and unpaired reads, as well as fragments with length > 200bp and Phred quality score < 20. Paired reads were merged into single interval of the fragment. BEDTools¹⁰³ were used to calculate bedgraphs of the genome coverage. IP and input bedgraphs of genome coverage were subsequently merged into multiple entry interval file of genome coverage. Number of DNA fragments coming from semi-synthetic nucleosome standards were counted for each barcode and IP efficiency was calculated for each histone mark.

$$BarcodeIPenrichment = \frac{\sum_1^n IP}{\sum_1^n input}$$

where, n is the identity of the n-th barcode assigned to the specific histone mark.

$$HMD(per/bp) = \frac{\left(\frac{IP}{input}\right) * 100\%}{BarcodeIPenrichment}$$

where, IP and input refer to the depth of the genome coverage at any given position of IP and input for specific histone mark. An IP efficiency can be interpreted as maximal yield of the IP for a given histone mark, or 100% HMD. However, there is a number of factors that can lead to

apparent HMD values greater than 100% including: uncertainty due to random sampling of sequenced fragments, in that case standard deviation is approximately equal to square root of sequencing depth, or off-target capture by antibody, where the off-target histone mark or combination of histone marks have greater IP efficiency than the antibody intended target mark. For all analyses, the HMD averaged over the N+1 and N+2 nucleosomes (taken to be 0 to +400bp into the gene body) was employed as representative of the promoter—this captures the most substantial H3K4me3 and H3K27me3 enrichment.

Genomic browser views were made using IGV. Heatmaps and gene ontology analysis was made using Homer Software¹⁰⁴. Further analysis and sectioning of data was conducted in R using the R code provided in Data Availability. Plots were made using ggplot2 and Microsoft Excel. For the bivalency tracks, *in silico* antibody off-specificity correction was performed as previously described for H3K9me3⁴⁶. ICeChIP-qPCR was performed with previously described primers⁴⁶.

Analysis of External Data

Bisulfite sequencing data was obtained from GEO series accession number GSE41923, dataset accession numbers GSM1027571, GSM1027572, GSM1027573, and GSM1027574. Methylation count files were obtained for each dataset and lifted to mm10. The average methylation for each promoter was then calculated for the 0 to +400bp region relative to the TSS of Refseq promoters using BEDTools.

Bulk RNA-seq data was obtained from GEO series accession numbers GSE108832 and GSE65697, dataset accession numbers GSM2913929, GSM2913930, GSM2913931, GSM1603282, GSM1603283, GSM1603284, GSM1603285, GSM1603286, and GSM1603287.

Pseudoalignment was conducted against the Refseq mm10 transcriptome using kallisto¹⁰⁵ with fragment length mean and standard deviation of 200 and 20, respectively, and 100 iterations. Pseudoalignments were then loaded into R for differential expression analysis using sleuth¹⁰⁶, with correction for batch effects between primed mESCs and NPCs due to contribution to principal components of the same. Differentially expressed genes were identified as $q \leq 0.05$. Single-cell RNA-seq data was obtained from GEO series accession number GSE113417 and aligned as above with kallisto.

Suz12 ChIP data to measure PCR2 localization for WT, Ezh2 KO, and Ezh1 KO/Ezh2 KO cells was obtained from GEO series accession number GSE116603, dataset accession numbers GSM3243624, GSM3243625, and GSM3243626. Peak files were obtained for all these datasets lifted to mm10. Ezh2 peaks were identified as peaks lost in Ezh2 KO relative to WT cells. Ezh1 peaks were identified as peaks lost in Ezh1 KO/Ezh2 KO relative to Ezh2 KO cells.

Set1A ChIP data was obtained from GEO series accession number GSE98988, dataset accession numbers GSM2629676, GSM2629677, GSM2629678, and GSM2629691. FastQ files were downloaded for the input and ChIP datasets for each replicate, then aligned to mm10 using Bowtie2 in end-to-end mode with the sensitive preset. Peak calling was then conducted on the alignments with MACS2¹⁰⁷, and consensus peaks for each replicate were identified.

Mil2 ChIP data was obtained from GEO series accession number GSE78708, dataset accession number GSM2073022. Peaks were obtained and lifted to mm10.

Methyltransferase assays

Enzymatic complexes were procured from Reaction Biology Corporation. Methyltransferase reactions were done using following concentrations of the enzymatic complexes: 200 nM hsMLL1(3745-3969), 200 nM hsMLL2(5319-5537), 400 nM hsMLL3(4689-4911), 200 nM hsMLL4(2490-2715), 800 nM hsSet1A(1418-1707), 800 nM hsSet1B(1629-1923), in a complex with hsWDR5(22-334), haRbBP5(1-538), hsAsh2L(2-534), 2x(hsDPY-30(1-99)), supplemented with 4%(v/v) RBC MLL enhancer (Reaction Biology Corp); 800 nM hsEzh1(2-747), 120 nM hsEzh2(2-746), in a complex with hsAEBP2(2-517), hsEED(2-441), hsRbAp48(2-425) and hsSUZ12(2-739) supplemented with 3.6mM hsJarid2(119-574) provided by Dr. Peter Lewis's laboratory. 30 ng/μl of semi-synthetic nucleosome substrate, 10 μM [³H]-SAM (50-80 Ci/mmol, Perkin Elmer Health Sciences), and enzymatic complexes were mixed in the Reaction Buffer (50 mM Tris pH 8.0, 91 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 10% glycerol, 1 mM PMSF) and incubated at 30°C. At designated time points, 4 μl of reactions were spotted on P81 Ion Exchange Cellulose Chromatography Paper (Reaction Biology Corp). Spotted paper was washed 4 times with 250 ml of 50 mM NaHCO₃ pH 9.0, for 5 minutes on a platform shaker, briefly washed with acetone, air-dried and immersed in scintillation fluid. ³H decay rate was measured by scintillation counter (LS 6000IC, Beckman).

Data and Software Availability

ICeChIP-seq data generated for this study has been deposited at the Gene Expression Omnibus (GEO) under accession numbers GSE108747 and GSE183155. R markdown file for analysis and sectioning of datasets is provided at <https://www.github.com/shah-rohan/bivalency/>.

1106 **Supplementary References**

- 1107 81. Young, N. L. *et al.* High Throughput Characterization of Combinatorial Histone Codes.
1108 *Mol. Cell. Proteomics MCP* **8**, 2266–2284 (2009).
- 1109 82. Lavarone, E., Barbieri, C. M. & Pasini, D. Dissecting the role of H3K27 acetylation and
1110 methylation in PRC2 mediated control of cellular identity. *Nat. Commun.* **10**, 1679 (2019).
- 1111 83. Radman-Livaja, M. & Rando, O. J. Nucleosome positioning: how is it established, and
1112 why does it matter? *Dev. Biol.* **339**, 258–266 (2010).
- 1113 84. Ruthenburg, A. J. *et al.* Recognition of a mononucleosomal histone modification pattern
1114 by BPTF via multivalent interactions. *Cell* **145**, 692–706 (2011).
- 1115 85. Rothbart, S. B. *et al.* Multivalent histone engagement by the linked tandem Tudor and
1116 PHD domains of UHRF1 is required for the epigenetic inheritance of DNA methylation. *Genes*
1117 *Dev.* **27**, 1288–1298 (2013).
- 1118 86. Savitsky, P. *et al.* Multivalent Histone and DNA Engagement by a PHD/BRD/PWWP
1119 Triple Reader Cassette Recruits ZMYND8 to K14ac-Rich Chromatin. *Cell Rep.* **17**, 2724–2737
1120 (2016).
- 1121 87. Yun, M., Wu, J., Workman, J. L. & Li, B. Readers of histone modifications. *Cell Res.* **21**,
1122 564–578 (2011).
- 1123 88. Amemiya, H. M., Kundaje, A. & Boyle, A. P. The ENCODE Blacklist: Identification of
1124 Problematic Regions of the Genome. *Sci. Rep.* **9**, 9354 (2019).
- 1125 89. Margueron, R. *et al.* Role of the polycomb protein EED in the propagation of repressive
1126 histone marks. *Nature* **461**, 762–767 (2009).

- 1127 90. Yuan, W. *et al.* Dense Chromatin Activates Polycomb Repressive Complex 2 to Regulate
1128 H3 Lysine 27 Methylation. *Science* **337**, 971–975 (2012).
- 1129 91. Hooper, M., Hardy, K., Handyside, A., Hunter, S. & Monk, M. HPRT-deficient (Lesch–
1130 Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature* **326**, 292–
1131 295 (1987).
- 1132 92. Conti, L. *et al.* Niche-Independent Symmetrical Self-Renewal of a Mammalian Tissue
1133 Stem Cell. *PLOS Biol.* **3**, e283 (2005).
- 1134 93. Abranches, E. *et al.* Neural Differentiation of Embryonic Stem Cells In Vitro: A Road
1135 Map to Neurogenesis in the Embryo. *PLOS ONE* **4**, e6286 (2009).
- 1136 94. Chen, Z., Grzybowski, A. T. & Ruthenburg, A. J. Traceless semisynthesis of a set of
1137 histone 3 species bearing specific lysine methylation marks. *Chembiochem Eur. J. Chem. Biol.*
1138 **15**, 2071–2075 (2014).
- 1139 95. Luger, K., Rechsteiner, T. J. & Richmond, T. J. Preparation of nucleosome core particle
1140 from recombinant histones. in *Methods in Enzymology* vol. 304 3–19 (Academic Press, 1999).
- 1141 96. Dyer, P. N. *et al.* Reconstitution of nucleosome core particles from recombinant histones
1142 and DNA. *Methods Enzymol.* **375**, 23–44 (2004).
- 1143 97. Lowary, P. T. & Widom, J. New DNA sequence rules for high affinity binding to histone
1144 octamer and sequence-directed nucleosome positioning. *J. Mol. Biol.* **276**, 19–42 (1998).
- 1145 98. Ullah, R. *et al.* Activity of the Human Rhinovirus 3C Protease Studied in Various
1146 Buffers, Additives and Detergents Solutions for Recombinant Protein Production. *PLOS ONE*
1147 **11**, e0153436 (2016).

1148 99. Cordingley, M. G., Callahan, P. L., Sardana, V. V., Garsky, V. M. & Colonno, R. J.
1149 Substrate requirements of human rhinovirus 3C protease for peptide cleavage in vitro. *J. Biol.*
1150 *Chem.* **265**, 9062–9065 (1990).

1151 100. Beckett, D., Kovaleva, E. & Schatz, P. J. A minimal peptide substrate in biotin
1152 holoenzyme synthetase-catalyzed biotinylation. *Protein Sci. Publ. Protein Soc.* **8**, 921–929
1153 (1999).

1154 101. Bailey, L. J. *et al.* Applications for an engineered Protein-G variant with a pH
1155 controllable affinity to antibody fragments. *J. Immunol. Methods* **415**, 24–30 (2014).

1156 102. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient
1157 alignment of short DNA sequences to the human genome. *Genome Biol.* **10**, R25 (2009).

1158 103. Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing
1159 genomic features. *Bioinforma. Oxf. Engl.* **26**, 841–842 (2010).

1160 104. Heinz, S. *et al.* Simple Combinations of Lineage-Determining Transcription Factors
1161 Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. *Mol. Cell* **38**,
1162 576–589 (2010).

1163 105. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq
1164 quantification. *Nat. Biotechnol.* **34**, 525–527 (2016).

1165 106. Pimentel, H., Bray, N. L., Puente, S., Melsted, P. & Pachter, L. Differential analysis of
1166 RNA-seq incorporating quantification uncertainty. *Nat. Methods* **14**, 687–690 (2017).

1167 107. Zhang, Y. *et al.* Model-based Analysis of ChIP-Seq (MACS). *Genome Biol.* **9**, R137
1168 (2008).

1169

Fig. 1 | Workflow and evaluation of reICeChIP-seq. (a) Schematic of reICeChIP-seq. The recombinant α -H3K4me3 Fab 304M3-B achieves high affinity by “clasping” the histone tail between two Fab molecules⁵³, a binding mode readily achieved by multiple copies of the Fab presented on a bead, but not by the Fab in solution. Thus, protease cleavage not only elutes nucleosomes from the beads but also likely from the Fab complex. (b) Enrichment of different barcoded nucleosomes in reICeChIP-seq (n=3 biological replicates). Error bars represent S.D. (c) Representative line plot showing histone modification density of H3K4me3, H3K27me3, and bivalency ICeChIP-seq presented with 95% confidence intervals (lighter shade) and input read depth in naïve mESCs. Bivalency is calibrated to the trans-bivalency nucleosome standard and corrected for off-target H3K9me3 pulldown.

Fig. 2 | Bivalency is widespread and does not resolve over differentiation. (a) Bivalency, H3K4me3, and H3K27me3 at all Refseq promoters in naive mESCs, with relative enrichment of GO terms. Genes are rank ordered by bivalency HMD at promoter, defined as the region from 0 to +400 bp relative to the TSS. (b) Representative locus view of H3K4me3, H3K27me3, and bivalency at promoters in naive mESCs (top), primed mESCs (centre), and NPCs (bottom), presented on the same scale of 0-125% HMD. (c) Number of promoters with bivalency HMDs above the given thresholds in each cell type out of a total of 42,622 Refseq promoters. (d) Metaprofiles of H3K4me3, H3K27me3, and bivalency at all promoters in naive mESCs, primed mESCs, and NPCs. Heatmaps for primed mESCs and NPCs are presented in Extended Data Fig. 3b. (e) Distribution of bivalency HMDs at all Refseq promoters in three cell states, zoomed to below 125% HMD. Overall, 99.5% of naive promoters, 87.3% of primed promoters, and 91.6% of NPC promoters have an HMD below 100%. Full plot in Extended Data Fig. 3a. (f) Metaprofiles of H3K4me3, H3K27me3, and bivalency at promoters identified as bivalent in naive mESCs (25% HMD threshold), tracked from naive mESCs to primed mESCs to NPCs. Heatmaps for bivalency are presented in Extended Data Fig. 3f. (g-h) Alluvial plots of dominance and bivalency of genes from (g) naive mESCs to NPCs or (h) primed mESCs to NPCs. Bivalency [$>25\%$ HMD] can be subcategorized into dominance classes by independent ICeChIP for the constituent marks, with H3K27me3 in excess ($H3K27me3/H3K4me3 > e^1$), H3K4me3 dominant ($H3K27me3/H3K4me3 < e^{-1}$), or intermediate ratios (no dominance). (i) Bivalency metaprofiles for gene subsets indicated in panel (h) from -3kb to +3kb relative to the TSS. **** $p < 2.2 \times 10^{-16}$.

Fig. 3 | Bivalency is neither sensitive nor specific for poised nor developmental genes. (a) Violin plots of gene expression⁷⁹ for all genes in naïve mESCs, non-bivalent genes (<25% HMD) in naïve mESCs, and bivalent genes (>25% HMD) tracked from naïve mESCs to the same genes in the indicated lineages. Significance computed by Welch's two-tailed *t*-test. (b) Gene expression vs. HMD for H3K4me3, H3K27me3, and bivalency (genes are binned into HMD deciles). (c) Proportion of actively transcribing cells by single-cell RNA-seq⁸⁰ vs. HMD for H3K4me3, H3K27me3, and bivalency (genes are binned into HMD deciles). (d) Sensitivity and specificity (Supplementary Note 5) of bivalent and non-bivalent genes in naïve mESCs identifying differentially expressed genes (DEGs) from the naïve state to the NPC state. (e) Metaprofiles of H3K4me3, H3K27me3, and bivalency and (f) heatmaps of bivalency in naïve mESCs at DEGs and non-DEGs relative to NPCs. (g) Sensitivity and specificity of bivalent and non-bivalent genes in primed mESCs identifying DEGs from the primed state to the NPC state. (h) Metaprofiles of H3K4me3, H3K27me3, and bivalency in primed mESCs at DEGs

and non-DEGs. **(i)** Gene ontology term enrichment of H3K27me3-dominant bivalent genes, H3K4me3-dominant bivalent genes, or bivalent genes with no clear dominance (q-value two-tailed Fisher hypergeometric test). **(j)** Metaprofiles of H3K4me3, H3K27me3, and bivalency in naïve mESCs at developmental and metabolic genes. **(k)** Gene ontology term enrichment of genes following the classic bivalency model: DEGs that lose bivalency from naïve mESCs (>25% HMD) to NPCs (<10% HMD). Significance computed by two-tailed Fisher hypergeometric test. * $q < 0.05$. ** $q < 0.01$. **** p or $q < 2.2 \times 10^{-16}$.

Fig. 4 | Bivalency does not provide appreciably more information than H3K4me3 and H3K27me3 alone for DEG prediction. (a) Receiver operator characteristic (ROC) curves for identifying DEGs from naïve mESCs to NPCs by H3K4me3, H3K9me3, H3K27me3, bivalency, $\ln(\text{H3K27me3}/\text{H3K4me3})$, or DNA methylation in naïve mESCs. For each point, parameter value threshold used to compute true positive rate (TPR) and false positive rate (FPR) is indicated by the colour. Traits with thresholds identifying non-DEGs rather than DEGs are marked with “rev.” (b) Legend for generalized linear models (GLMs) in panels c-d. (c) Accuracy of trivial model and GLMs by threshold accuracy (gene identified as DEG if logistic regression > 0.5; left) and by ROC area under curve (right). (d) ROC curves for identifying DEGs from naïve mESCs to NPCs by different GLMs. For each point, logistic regression threshold value used to compute TPR and FPR is indicated by the colour.

Extended Data Fig. 1 | Evaluation of sequential ChIP methods. (a) Enrichment of on- and off-target nucleosome standards under sequential ChIP protocols developed by Bernstein et al.¹⁶, Voigt et al.⁴⁹, and Seenundun et al.²⁹. (b-c) Enrichment at different sequential ICEChIP steps with (b) chemical denaturant elution and (c) immunoglobulin and serum elution. (d) Enrichment of different nucleosome standards with ICEChIP-qPCR performed against H3K4me3, H3K27me3, and bivalency, with beads showing very little retention of chromatin (n=3 technical replicates). Error bars represent standard deviation. (e) Different configurations of bivalency on a single nucleosome. Of these, only trans-bivalency has been identified by mass spectrometry^{49,81}.

Extended Data Fig. 2 | Evaluation of reICEChIP specificity and standards. (a) Representative genome browser view of H3K4me3, H3K27me3, and bivalency, shown as a range of possible values by normalization to trans-bivalent (upper limit) or cis-bivalent (lower limit) nucleosome standards. **(b)** Relative pulldown of different nucleosome standards in ICEChIP-seq, normalized to the most-enriched standard. **(c)** Scatterplots of reads from DNA barcodes applied to nucleosome standards in ICEChIP-seq. **(d)** Violin plots of peak breadth (consecutive segment of 50bp windows overlapping promoter with >25% HMD) for H3K4me3 (green) and bivalency (blue) at non-bivalent and bivalent genes (>25% HMD) in naïve mESCs. **(e-f)** Autocorrelation of (e) H3K4me3 and (f) bivalency HMDs between nucleosomes in naïve mESCs. Nucleosomes are defined as sequential 200bp windows from the TSS.

Extended Data Fig. 3 | Tracking bivalent genes through differentiation. (a) Distribution of bivalency HMDs at all Refseq promoters in three cell states. (b) Heatmaps of bivalency at all Refseq promoters in primed mESCs and NPCs. Genes are ordered by bivalency HMD at the promoter. (c) Venn diagram showing overlap of bivalent genes (25% HMD threshold) in naïve mESCs, primed mESCs, and NPCs. (d-e) Metaprofiles of H3K4me3, H3K27me3, and bivalency for bivalent genes in naïve mESCs with a (d) 10% or (e) 50% HMD threshold. (f) Heatmaps and metaprofiles of bivalent genes from naïve mESCs. (g) Metaprofiles of H3K4me3, H3K27me3, and bivalency at genes tracked from primed mESCs to NPCs for bivalent genes in primed mESCs (>25% HMD). (h) Heatmaps and metaprofiles of bivalent genes in primed mESCs that are not bivalent in naïve mESCs.

Extended Data Fig. 4 | Comparing our bivalent genes to other studies. (a-b) Contingency tables and meta-profiles for genes that are identified as >25% bivalent in our study and by Mikkelsen et al.¹⁸, Mas et al.³⁵, and Xiao et al.⁵⁸, wherein: (a) gene is identified as bivalent in the external study if overlapping H3K4me3 and H3K27me3 peaks overlap the 0 to +400bp region of a gene relative to the TSS, or (b) gene is identified as bivalent in the external study if overlapping H3K4me3 and H3K27me3 peaks overlap the region from 2.5kb upstream of the TSS to the end of the gene²². **(c)** Overlap of bivalent genes from external datasets (as defined in part a) with each of our bivalent gene dominance classes in naïve mESCs. Significance computed by two-tailed Fisher hypergeometric test. **(d)** Overlap of genes with bivalency HMD > 25% and with H3K4me3 + H3K27me3 HMD > 25% in all three cell states.

Extended Data Fig. 5 | Bivalency changes across differentiation by modification dominance class. (a-c) Metaprofiles of H3K4me3, H3K27me3, and bivalency for bivalent genes (>25% HMD) in naïve mESCs that are (a) H3K27me3 dominant ($H3K27me3/H3K4me3 > e^1$), (b) H3K4me3 dominant ($H3K27me3/H3K4me3 < e^1$), or (c) have no dominance in naïve mESCs, tracked through three cell states. **(d-e)** Metaprofiles of H3K4me3, H3K27me3, and bivalency for bivalent genes (>25% HMD) in primed mESCs that are for indicated dominance classes tracked from primed mESCs to NPCs.

Extended Data Fig. 6 | Methyltransferase assays identifying potential pathways for establishment of bivalency. (a-b) Methyltransferase assays for MLL1, MLL2, MLL3, MLL4, and Set1B core HMTase complexes using (a) 15 ng/uL (n=6) and (b) 20 ng/uL (n=5) semisynthetic nucleosomes as substrates for methylation. Endpoints were established at 180 min by kinetic evaluation to be sensitive to difference in activity for this panel. Signal is corrected for background and no nucleosome substrate activity. Error bars represent standard deviation.

Extended Data Fig. 7 | HMTase peaks and bivalency. Contingency tables and metagene profiles in naïve mESCs for genes with and without overlapping HMT peaks. Ezh1 and Ezh2 peaks were identified as Suz12 peaks lost upon Ezh1 or Ezh2 knockout⁸². Set1A peaks were identified by ChIP against Set1A⁶². Mll2 peaks were identified by ChIP against Mll2⁴⁵.

Extended Data Fig. 8 | Bivalency and differential gene expression. (a-b) Violin plots of gene expression for (a) all genes and (b) bivalent (>25% HMD) genes in each cell state. **(c)** Bivalency metaprofiles in naïve mESCs at promoters binned by gene expression decile. **(d)** Violin plots of bivalency HMD in naïve mESCs at promoters with and without CpG islands. Inset shows proportion of genes that are bivalent in sets of genes classified by CpG content: high-CpG promoters (HCP), intermediate-CpG promoters (ICP), and low-CpG promoters

(LCP), defined as previously described by Mikkelsen et al.¹⁸. Total number of genes in each class is provided as n. **(e)** Violin plots of DNA methylation at bivalent and non-bivalent genes (top), broken by dominance class for bivalent genes (bottom). **(f-g)** Violin plots of gene expression in (f) non-bivalent (<25% HMD) and (g) bivalent (>25% HMD) genes from naïve mESCs that are H3K27me3 dominant ($H3K27me3/H3K4me3 > e^1$; left), have no clear dominance (centre), or are H3K4me3 dominant ($H3K27me3/H3K4me3 < e^1$; right). **(h-k)** Metaprofiles of H3K4me3, H3K27me3, and bivalency at genes tracked from naïve mESCs to primed mESCs to NPCs for (h) DEGs, (i) non-DEGs, (j) genes upregulated from naïve mESCs to NPCs, and (k) genes downregulated from naïve mESCs to NPCs. **** $p < 10^{-16}$ (Welch's two-tailed t-test).

Extended Data Fig. 9 | Bivalency at different classes of genes. (a) Metaprofiles of H3K4me3, H3K27me3, and bivalency at genes tracked from naïve mESCs to primed mESCs to NPCs for bivalent genes of indicated gene ontology terms. **(b)** Metaprofiles of H3K4me3, H3K27me3, and bivalency at genes tracked across differentiation for genes that lose bivalency at the promoters (0 to +400bp relative to TSS) from naïve mESCs (>25% HMD) to NPCs (<10% HMD) and are upregulated (top) or downregulated (bottom) over differentiation.

Extended Data Fig. 10 | Modelling the additional information content provided by bivalency over H3K4me3 and H3K27me3 alone. (a) ROC curves for identifying DEGs from primed mESCs to NPCs by H3K4me3, H3K9me3, H3K27me3, bivalency, ln(H3K27me3/H3K4me3), or DNA methylation in primed mESCs. For each point, parameter value threshold used to compute true positive rate (TPR) and false positive rate (FPR) is indicated by the colour. Traits with thresholds identifying non-DEGs rather than DEGs are marked with “rev.” (b) Bayes Information Criterion (BIC) for logistic models identifying DEGs from naïve mESCs or primed mESCs to NPCs with different parameters. (c) Legend for generalized linear models (GLMs). (d) Accuracy of trivial model and GLMs by threshold accuracy (gene identified as DEG if logistic regression > 0.5; left) and by ROC area under curve (right). (e) ROC curves for identifying DEGs from primed mESCs to NPCs by different GLMs. For each point, logistic regression threshold value used to compute TPR and FPR is indicated by the colour.