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Citation: Lesch, Bluma J, Sherman J Silber, John R McCarrey, and David C Page. "Parallel Evolution of Male Germline Epigenetic Poising and Somatic Development in Animals." Nature Genetics 48, no. 8 (June 13, 2016): 888–894.

As Published: http://dx.doi.org/10.1038/ng.3591

Publisher: Springer Nature

Persistent URL: http://hdl.handle.net/1721.1/107718

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

Parallel evolution of male germline epigenetic poising and somatic development in animals

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Abstract

Changes in gene regulation frequently underlie changes in morphology during evolution, and differences in chromatin state have been linked with changes in anatomical structure and gene expression across evolutionary time. Here, we assess the relationship between evolution of chromatin state in germ cells and evolution of gene regulatory programs governing somatic development. We examined the poised (H3K4me3/H3K27me3 bivalent) epigenetic state in male germ cells of five mammalian and one avian species. We find that core genes poised in germ cells of multiple amniote species are ancient regulators of morphogenesis that sit at the top of transcriptional hierarchies controlling somatic tissue development, while genes that gain poising in germ cells of individual species act downstream of core poised genes during development in a species-specific fashion. We propose that critical regulators of animal development gained an epigenetically privileged state in germ cells, manifested in amniotes by H3K4me3/H3K27me3 poising, early in metazoan evolution.

Introduction

Together, maternal and paternal germ cells provide all the information needed to initiate formation of a new embryo at fertilization. Along with a haploid genome, germ cells carry gene regulatory information, which guides gene expression during sperm or egg development[1,2] and may impact development of the embryo in the next generation[3-5]. Changes in gene regulation contribute to evolution of morphology across diverse species[6,7], and recent studies assessing chromatin state in specific tissues across multiple species have found that evolution of chromatin state is associated with evolution of gene expression and anatomical structure.[8-11] Building on this work, we reasoned that evolution of chromatin state in germ cells might be similarly associated with evolution of gene expression in somatic tissues of the embryo.

We focused our attention on evolution of epigenetic 'poising' in germ cells. Epigenetic poising is defined by the simultaneous presence of two opposing histone modifications, the activating mark H3K4me3 and the repressive mark H3K27me3, as well as by transcriptional repression[12-14]. It has been best studied in embryonic stem cells (ESCs), where it is associated with genes involved in lineage specification. In ESCs, the presence of the activating H3K4me3 mark is thought to 'poise' these otherwise silent genes for activation following receipt of a differentiation cue, while the H3K27me3 mark maintains repression in the pluripotent state[12]. Consistent with this model, in mouse embryos *in vivo*, *Hox* genes move sequentially from a poised state to an active H3K4me3-only state, concurrent with their activation in an anterior-to-posterior direction[15,16]. *Hox* genes and other developmental regulators are poised in mouse and human germ cells but are not expressed in developing gametes; rather, genes poised in germ cells are

expressed in somatic tissues during embryogenesis[17-20]. We hypothesized that evolution of epigenetic poising in mammalian germ cells reflects the evolution of a transcriptional program controlling somatic gene expression and morphogenesis in embryos.

Evaluating this hypothesis demanded we carry out four tasks: characterize the poised state in the germ cells of multiple mammalian species; relate conservation of germ cell poising to conservation of developmental function in mammals; define the relationship between differences in germ cell poising and differences in developmental function among specific evolutionary lineages; and reconstruct the evolutionary origins of these relationships by comparison to non-mammalian taxa.

We used comparative epigenetic profiling in mammalian germ cells to address these questions. We examined genome-wide expression, H3K4me3, and H3K27me3 data in male germ cells of five mammalian species spanning 175 million years of evolution. We found that evolution of poising in male germ cells parallels evolution of somatic gene expression and development in the embryo. We propose an ancient evolutionary relationship between germline chromatin and embryonic gene expression.

Results

Gene expression and chromatin state in male germ cells

We collected H3K4me3 and H3K27me3 ChIP-seq data, as well as RNA-seq data, in sorted male germ cells from five species spanning 175 million years of evolutionary divergence in the

mammalian lineage: human, rhesus macaque, mouse, bull, and opossum (Fig. 1a, Supplementary Fig. 1, and Supplementary Table 1). We obtained data from cells at two time points during male germ cell development: prophase of meiosis I (pachytene spermatocytes), and following completion of meiosis (round spermatids). These cell types are unique in that they can be identified and reliably collected from whole testes in multiple mammalian species without the use of transgenes or genetic markers (see Methods). In addition, they differ substantially from each other in their place in the cell cycle and in the physical state of their chromatin: pachytenes are tetraploid cells in meiotic prophase, with large nuclei and synapsed pairs of homologous chromosomes, while round spermatids are haploid cells that have completed meiosis and have small, compact nuclei. Inclusion of both cell types in our study allowed us to control for the effects of chromatin compaction and physical state of the nucleus during spermatogenic development.

Before turning to the poised state, we first examined the relationships between H3K4me3, H3K27me3, and expression datasets collected from different species. In all species, expression levels were positively correlated with H3K4me3 signal and negatively correlated with H3K27me3 signal, consistent with the known association of these marks with gene activity and repression, respectively (Fig. 1b, Supplementary Fig. 2). Our data included three biological replicates for human, two for rhesus, mouse, and opossum, and one for bull (Supplementary Fig. 3); biological replicates were highly similar as evaluated by principal component analysis (Supplementary Fig. 4a) or by hierarchical clustering (Fig. 1c, Supplementary Fig. 4b). For H3K4me3, clustering accurately separated pachytene spermatocytes from round spermatids within each species, but did not fully recapitulate phylogenetic relationships, as has been

previously reported for the H3K4me3 mark in other tissues[10]. In contrast, we derived correct or nearly-correct phylogenies from both expression and H3K27me3 data. For expression, H3K4me3, and H3K27me3 data, greater dissimilarity between samples corresponded to greater evolutionary divergence between species (Fig. 1d, Supplementary Note 1).

Identification of poised chromatin in male germ cells

To identify genes associated with poised chromatin in germ cells of each species, we calculated read counts in four-kilobase intervals surrounding the transcription start sites of annotated genes, after normalizing for library size and subtracting input signal (Supplementary Files 1-10). Throughout our analysis, we considered only genes with orthologs in all five species (a total of 14,362 orthology groups). Genes above a threshold of 0.5 reads per million for H3K4me3 and H3K27me3 signal, and whose expression level was equal to or less than 5 FPKM, were called as poised. Within each species, we further filtered for genes at which the poised state was retained in both pachytene spermatocytes and round spermatids, implying that it is stable across much of spermatogenic development. Stably-poised gene sets identified in this manner were robust to changes in ChIP and expression threshold (Supplementary Figs. 5 and 6).

This approach identified 1,200-3,600 poised genes in each species (Fig. 2a,b and Supplementary Table 2). We verified that co-occurrence of high H3K4me3 and H3K27me3 signals at poised genes represented the simultaneous presence of the two marks on the same DNA molecule, not heterogeneity of chromatin state within our cell population, by performing sequential ChIP at the promoters of representative poised genes in both mouse (Fig. 2c and Supplementary Fig. 7a) and opossum (Fig. 2d and Supplementary Fig. 7b) round spermatids. We confirmed that four out of

four mouse and two out of two opossum poised promoters were simultaneously marked by both H3K4me3 and H3K27me3, demonstrating that these genes are marked by a *bona fide* poised state in round spermatids. In general, between one quarter and three quarters of poised genes were shared between any two species (Supplementary Table 3), and dissimilarity in poising was positively correlated with evolutionary divergence time (Fig. 2e). Regardless of evolutionary distance, overlap in poised gene sets between each species pair was greater than expected by chance ($p < 10^{-15}$ for all pairs, Fisher's exact test).

Conserved poising at developmental regulators in mammals

Four hundred and five genes were poised in all five species ($p < 10^{-280}$ compared to expected for five-way overlap, see Methods) (Fig. 3a and Supplementary Table 4). These genes were well distributed across chromosomes (Supplementary Fig. 8a). At the sequence level, the promoter regions of these genes were significantly better conserved than those of human-specific poised genes ($p < 10^{-14}$, Welch t-test) or genes with conserved retention of H3K27me3 but not necessarily H3K4me3 ($p < 10^{-4}$, Welch t-test) (Supplementary Fig. 8b). The set of genes poised in germ lines of all five mammalian species, henceforth referred to as 'core' poised genes, was strongly enriched for genes encoding transcription factors, with a particularly striking enrichment for homeodomain-containing transcription factors (Fig. 3b and Supplementary Fig. 8c).

We used pre-defined Gene Ontology (GO) categories to confirm enrichment of transcription factor-encoding genes in the core poised gene set. The 405 core poised genes were significantly enriched for genes belonging to the GO category "sequence specific DNA binding transcription

131 factor activity" (GO:0003700) compared to all genes with five-way orthologs, to human- or
132 mouse-specific poised genes, or to genes with conserved retention of H3K27me3 but not
133 necessarily H3K4me3 (Fig. 3c).
134
135 We then asked whether, in addition to encoding proteins with a shared molecular function as
136 sequence-specific transcription factors, the set of core poised genes had a unifying biological
137 function during development. We examined enrichment of GO biological function categories in
138 the set of core poised genes. We found that enriched GO categories described processes
139 involved in patterning and organ formation, including "embryonic organ morphogenesis",
140 "anterior/posterior pattern specification", "limb development", and "gastrulation" (Fig. 3d and
141 Supplementary Table 5). Core poised genes were not enriched for germ cell-related functions,
142 such as meiosis, spermatid development, or sperm maturation. These findings imply that the
143 core poised genes have a shared biological role, specifically, transcriptional regulation of body
144 patterning and somatic tissue specification during embryogenesis.
145
146 If the core poised genes are involved in patterning and tissue specification, they should be
147 expressed during an interval in embryogenesis when these processes are occurring. We queried
148 the MGI Gene Expression Database[21], which contained expression data for 13,837 mouse genes
149 at the time of our study, to determine the interval when each of these genes is first expressed.
150 Core poised genes were enriched for expression during embryogenesis compared to other genes
151 with orthologs in all five species; this difference was especially evident between gastrulation and
152 the end of somite formation (Thieler stage [TS] 11-22, Fig. 3e). A subset of core poised genes
153 involved in trophectoderm specification (e.g. *Cdx2* [*Caudal type homeobox 2*], *Hand1* [*Heart*

and neural crest derivatives expressed transcript 1], and *Tpbg* [*Trophoblast glycoprotein*]) was also expressed early in embryogenesis (TS 2-4)[22].

We then examined specific cases where the regulatory hierarchies involved in body part and organ field specification are well defined, and found that core poised genes are central to these processes. Core poised genes sit at the top of such specification hierarchies, including *PTF1A* (*Pancreas specific transcription factor 1a*) and *PDX1* (*Pancreatic and duodenal homeobox 1*) in pancreatic development[23]; *EN1/2* (*Engrailed 1* and *2*)*, OTX1* (*Orthodenticle homeobox 1*), *LMX1A* (*LIM homeobox transcription factor alpha*) and *GBX2* (*Gastrulation brain homeobox 2*) in cerebellar development[24]; *NKX2-5* (*NK homeobox 2-5*), *HAND1* and *HAND2* (*Heart and neural crest derivatives expressed transcript 2*) in heart development[25,26]; and *MSX1/2* (*Msh homeobox 1* and *2*) and *NKX2-2* (*NK homeobox 2-2*) in neural tube regionalization[27].

To obtain quantitative support for this finding, we examined the connectedness of core poised genes compared to other genes in the context of three experimentally-supported developmental regulatory networks: pancreas[23], heart[25], and cerebellum[24]. Considering all three of these networks together, core poised genes had more regulatory connections than other genes (mean 4.50 compared to 2.27 connections, p=0.01765 [one-sided Mann-Whitney U test]). Core poised genes also exhibited greater network centrality (betweenness centrality, the likelihood that a particular gene lies on the shortest path connecting two other genes) compared to other genes upregulated during differentiation and specification stages in an *in vitro* model of human cortical development[28] ($p=1.43 \times 10^{-6}$, one-sided Mann-Whitney U test).

We conclude that the core poised genes constitute critical upstream regulators of gene expression during mammalian embryogenesis. Indeed, many of the core poised genes participate in developmental 'kernels', conserved genetic circuits controlling specification of body part progenitor fields[29,30]. Kernel architecture extends deep into the metazoan lineage and is highly conserved across metazoa, partly because extensive regulatory interactions among kernel constituents mean that perturbation of any one component can have catastrophic effects on body part patterning[29,30]. Indeed, we found that knockout alleles of 83 of the core poised genes (21%) resulted in embryonic lethality in the mouse, whereas knockout alleles of only 10% of all genes with orthologs in all five mammalian species resulted in the same phenotype ($p=3.77 \times 10^{-11}$, Fisher's exact test)[31].

Differences in germline poising between species

Given that genes with strongly conserved roles in metazoan development exhibit conservation of poising in germ cells, we wondered if genes that gain species-specific developmental functions might also acquire species-specific poising in germ cells. To address this question, we turned our attention to the five sets of genes poised specifically in only one of the five species evaluated ('differentially poised' genes), acknowledging that a subset of these genes may be mis-assigned as 'specific' due to false negative calls in one or more of the other four species (Fig. 4a,b Supplementary Table 6). None of the five sets of differentially poised genes was strongly enriched for GO developmental functions. However, where comparative expression data in specific developmental structures was available, species differences in germline poising were correlated with species differences in developmental expression[8,9,32,33]. For example, *ZSWIM4* and *LMF1*, which are poised specifically in human germ cells (Fig. 4a and Supplementary Fig.

9a), are expressed in human but not mouse or bovine placenta; likewise, *Ccrl2* and *Smug1* are poised specifically in mouse germ cells (Fig. 4b and Supplementary Fig. 9b) and expressed in mouse but not human or bovine placenta[32]. Similarly, *HIPK2* is poised specifically in human germ cells (Supplementary Fig. 9a) and has acquired a human-specific enhancer active during limb development[8].

We predicted that differential poising and differential expression during development in a given species would correspond to differences in regulatory sequence compared to orthologous genes in the other four species. To test this prediction, we searched for motifs enriched in the promoters of each of the five differentially poised gene sets relative to their non-poised orthologs (Supplementary Table 7). We found that motifs gained in poised promoters frequently corresponded to predicted binding motifs for transcription factors encoded by core poised genes (71% of human, 62% of rhesus, 51% of mouse, 33% of bull, and 60% of opossum gained motifs) (Fig. 4c, Supplementary Table 7). In general, motifs gained in differentially poised promoters were different in each species. Together with expression differences, these results imply that acquisition of epigenetic poising in germ cells may occur in parallel with gain of regulation by core poised genes during somatic development. Extension of germline poising to new developmental factors may facilitate their recruitment into ancient developmental circuits regulated by core poised genes.

We also identified cases of single-lineage losses, in which a gene was poised in all but one of the five species examined (Supplementary Table 8). As with single-lineage gains, some of these instances may be due to false-negative poised gene calls in one species. However, some

instances of single-lineage loss of poising are supported by previous reports of recent evolutionary divergence in expression or function of the associated gene. For example, of the 36 genes poised in four mammals but not in human, three are reported to have divergent expression patterns in human compared to other mammals (*AIM1*, *EPHA5*, and *THBS4*)[34-36], one is associated with differences in loss-of-function phenotype between human and mouse (*DOCK8*)[37], and three are associated with recent positive selection in the human lineage (*AIM1*, *COL11A1*, and *LYPD1*)[38-40]. Like lineage-specific gains, lineage-specific loss of poising in the germ line may therefore reflect recent lineage-specific changes in developmental regulation and function.

Conservation of germline poising beyond mammals

The set of core poised genes is notable both for its origins deep in the metazoan lineage and for its specificity to metazoa; 224 (55%) of the core poised genes have orthologs in the fly *Drosophila melanogaster* but not in the yeast *Saccharomyces cerevisiae*, compared to 36% of all genes with orthologs in all five mammals ($p = 9.59 \times 10^{-16}$, Fisher's exact test), implying that the majority of core poised genes arose before the divergence of protostomes and deuterostomes but after the divergence of animals from fungi. We asked when these genes might first have gained a specialized epigenetic state in the metazoan germ line.

First, we compared our mammalian data to a non-mammalian amniote, the chicken. Together with reptiles, birds constitute the closest living relatives of the mammalian clade (Fig. 1a)[41]. We collected ChIP- and RNA-seq data from chicken germ cells at time points matching those used for the five mammalian species (Supplementary File 11). Using identical filtering conditions, we

identified 1716 genes poised in the chicken germ line (Supplementary Table 9). Of the 405 core poised genes we defined in mammals, 347 have orthologs in the chicken genome; of these, 215 (62%) are also poised in the chicken germ line. For the core poised genes in mammals, the set of genes whose orthologs were also poised in chicken was significantly enriched for sequence-specific transcription factors compared to the core poised genes that were not poised in chicken (Fig. 5a,b). We conclude that epigenetic poising of developmental transcriptional regulators in germ cells is at least as old as the amniote common ancestor, placing its origin more than 300 million years ago. In at least five cases, core poised genes that were poised in mammalian but not chicken germ cells could also be correlated to differences in development between mammals and birds[42-45], supporting the hypothesis that acquisition of poising in the germ line is related to acquisition of somatic developmental function. For example, *TPBG* is poised in all five mammals but not in chicken (Fig. 5c), consistent with its early expression in trophectoderm, a mammal-specific structure[45,46]. Given previous reports of a multivalent chromatin state comprised of multiple repressive and activating histone marks at developmental genes in zebrafish sperm[47], it will be interesting to trace the evolutionary history of the poised state in additional non-amniote vertebrate species.

Using the chicken data, we further examined the scenario in which genes whose poised state was shared in distantly related species were not poised in germ cells of intermediate lineages. Such a scenario implies either convergent evolution, requiring two independent epigenetic gains, or deep loss of poising followed by recent reacquisition, requiring a loss followed by a gain. Either explanation calls for at least two independent evolutionary events and is expected to be rarer than scenarios requiring either uninterrupted conservation (zero events) or single-lineage gains or

losses (one event). Indeed, among the 11,188 genes with orthologs in all six species, 211 were poised in human, rhesus, and mouse only, implying a single gain in the primate-rodent ancestor, compared to only 35 in human, opossum, and chicken or 14 in rhesus, opossum, and chicken, each requiring either convergence or deep loss in the placental lineage followed by a gain (Supplementary Table 9). For at least one gene among the 35 shared by human, opossum, and chicken (*NCS1*, *Neuronal calcium sensor 1*), expression patterns in human and chicken were more similar than in human and mouse, implying convergent evolution of expression.[48,49]

To examine the possibility that the origins of germline poising lie deeper in the metazoan lineage, we compared our set of core poised genes to Polycomb ChIP-microarray data in sorted *Drosophila* male germ cells[50]. We found that 5.2% of all genes whose promoters were marked by high levels of Polycomb in the *Drosophila* germ line were orthologs of the core poised genes, compared to 2.5% of genes with low Polycomb levels ($p=1.059 \times 10^{-5}$, Fisher's exact test). Overall, orthologs of core poised genes were enriched for Polycomb signal in the *Drosophila* germ line (Supplementary Fig. 10a). This effect is modest, but suggests that orthologs of some mammalian core poised genes may have acquired a specialized epigenetic state, characterized by Polycomb binding and H3K27me3, in germ cells before the emergence of the bilaterian ancestor[51], and retained it independently in protostomes and deuterostomes (Fig. 5d).

Discussion

We show here that evolution of epigenetic poising in male germ cells is closely linked to evolution of somatic gene expression in developing mammalian embryos. Germline poising is

conserved throughout the mammalian lineage at genes that are central to the transcriptional networks governing somatic development, and individual genes recruited to these networks in specific lineages also gain poising in germ cells. Poising of central developmental genes in male germ cells is at least as old as the amniote common ancestor, placing its origin at least 300 million years ago. Such deep conservation implies a functional role for the poised state in germ cells.

It is easy to envision a role for H3K27me3 at somatic genes in the germ line: this repressive mark reinforces silencing of genes whose expression in germ cells would disrupt their function and identity. However, we found that genes exhibiting conservation of H3K27me3 without H3K4me3 do not show the same functional enrichments as genes with conserved poising (Fig. 3c and Supplementary Table 5), indicating that H3K27me3 alone does not play the same biological role as H3K27me3/H3K4me3 bivalency. H3K4me3 may play a protective role at poised promoters as an antagonist of DNA methylation[5,52]. It is also possible that H3K4me3 helps to prepare poised genes for expression in somatic tissues following fertilization, similar to its proposed role in ESCs. Consistent with this hypothesis, altered regulation of H3K4 methylation state in developing male germ cells in the mouse perturbs somatic tissue development in embryos of the next generation[3]. The detailed mechanism by which this epigenetic information might be transmitted through fertilization remains unclear: modified histones may be carried in mature spermatozoa[19,20,53], or poised sites may be marked by an RNA or protein intermediate in sperm and re-established in the early embryo. We found that published ChIP-seq data from mature mouse[20] and human[19] spermatozoa is consistent with

retention of modified histones at core poised genes (Supplementary Fig. 10b), but this finding does not exclude the participation of additional factors in marking poised sites.

Our study leverages comparative analysis of *in vivo* epigenomic data across multiple species to identify a set of genes that is epigenetically privileged in the mammalian germ line. This privileged state is manifested by H3K4me3/H3K27me3 bivalency in amniotes, and association of H3K27me3 with core members of this gene set extends deep in animal evolution to the common bilaterian ancestor. Future work in additional animal species will be important to better define the evolutionary history of this privileged epigenetic state in the metazoan germ line.

Together with existing studies from non-amniote species[27,50,54-58], our data implicate core poised genes as ancient regulators of metazoan development that sit at the heart of somatic developmental networks, and differentially poised genes as agents of lineage-specific change. In mammalian germ cells, the poised state thus represents a memory of ancient developmental regulatory hierarchies and a device for understanding their evolution.

URLs

http://hannonlab.cshl.edu/fastx_toolkit/index.html

http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

http://www.informatics.jax.org/gxd

http://www.informatics.jax.org/allele

http://www.R-project.org/

Accession codes

ChIP-seq and RNA-seq data are available at the Sequence Read Archive (SRA) under accession code SRP057141 and at the Gene Expression Omnibus (GEO) under accession code GSE68507.

Acknowledgments

This project was funded by an HHMI award to DCP, by a Hope Funds for Cancer Research postdoctoral fellowship to BJL, and by a Burroughs-Wellcome Career Award to BJL. We thank H. Skaletsky for statistical advice and analysis; R. Young for advice on ChIP-seq analysis and critical reading of the manuscript; and P. Reddien for critical reading of the manuscript.

Author contributions

B.J.L. designed the project, conducted experiments, analyzed data, and wrote the paper. D.C.P. designed the project and wrote the paper. S.J.S. provided human testis samples and contributed to writing the paper. J.R.M. isolated germ cells for all samples and contributed to writing the paper.

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Figures

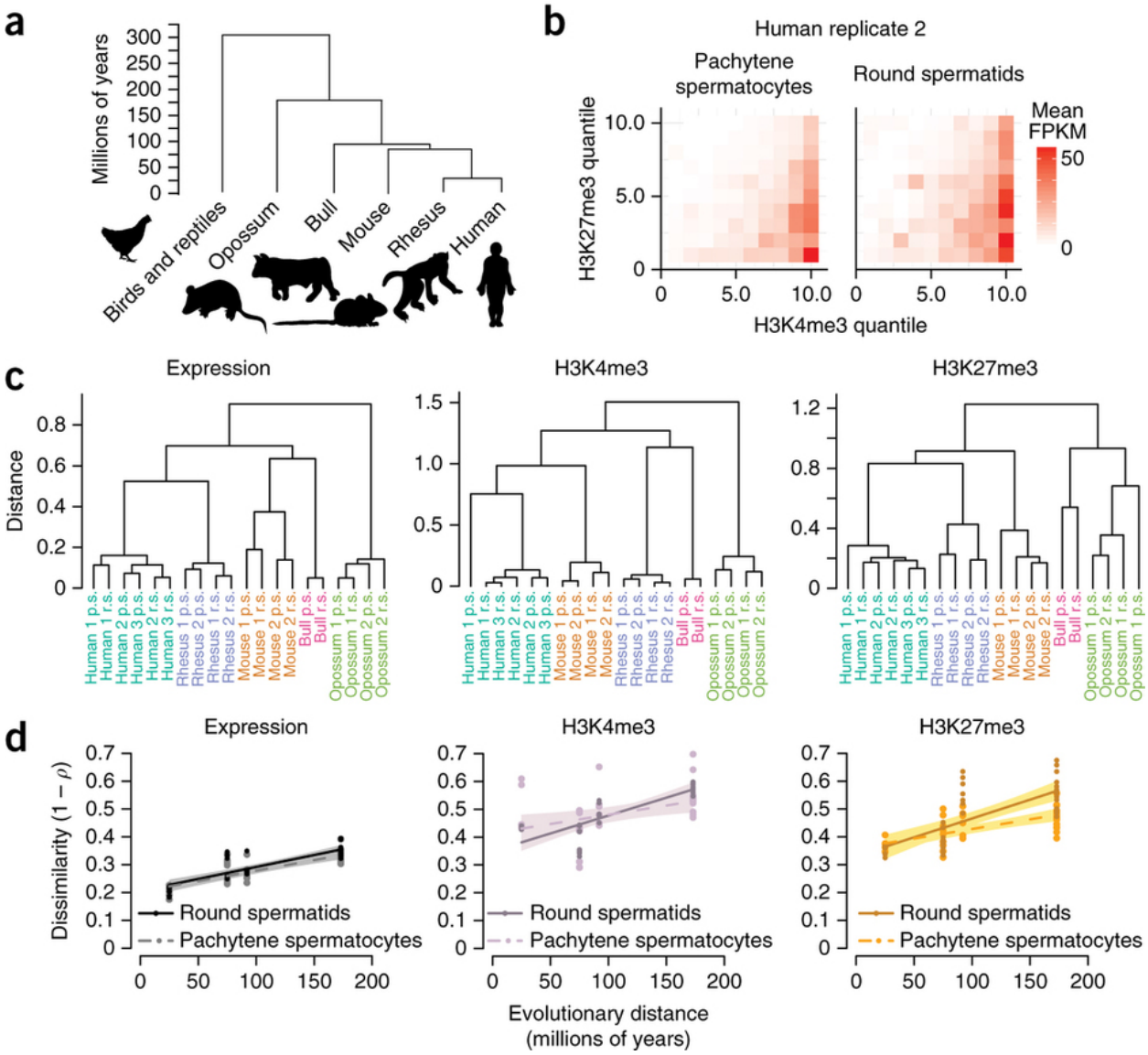

Figure 1. Gene expression and chromatin state in the mammalian germ line. **a**, Phylogeny of the mammalian species included in this study. **b**, Heat maps showing mean gene expression level as a function of H3K4me3 and H3K27me3 quantile in human pachytene spermatocytes and round spermatids. Similar heat maps for all samples are shown in Supplementary Figure 2. **c**, Hierarchical clustering of datasets by expression, H3K4me3, or H3K27me3 using 1-ρ (ρ,

Spearman's correlation coefficient) as a distance metric. p.s., pachytene spermatocytes; r.s., round spermatids. **d**, Divergence in expression, H3K4me3, and H3K27me3 for pachytene spermatocytes and round spermatids as a function of evolutionary distance, using 1-ρ as a dissimilarity metric. Lines represent best linear fit to pachytene spermatocyte (dashed) and round spermatid (solid) data. The shaded area surrounding each line indicates 95% confidence interval.

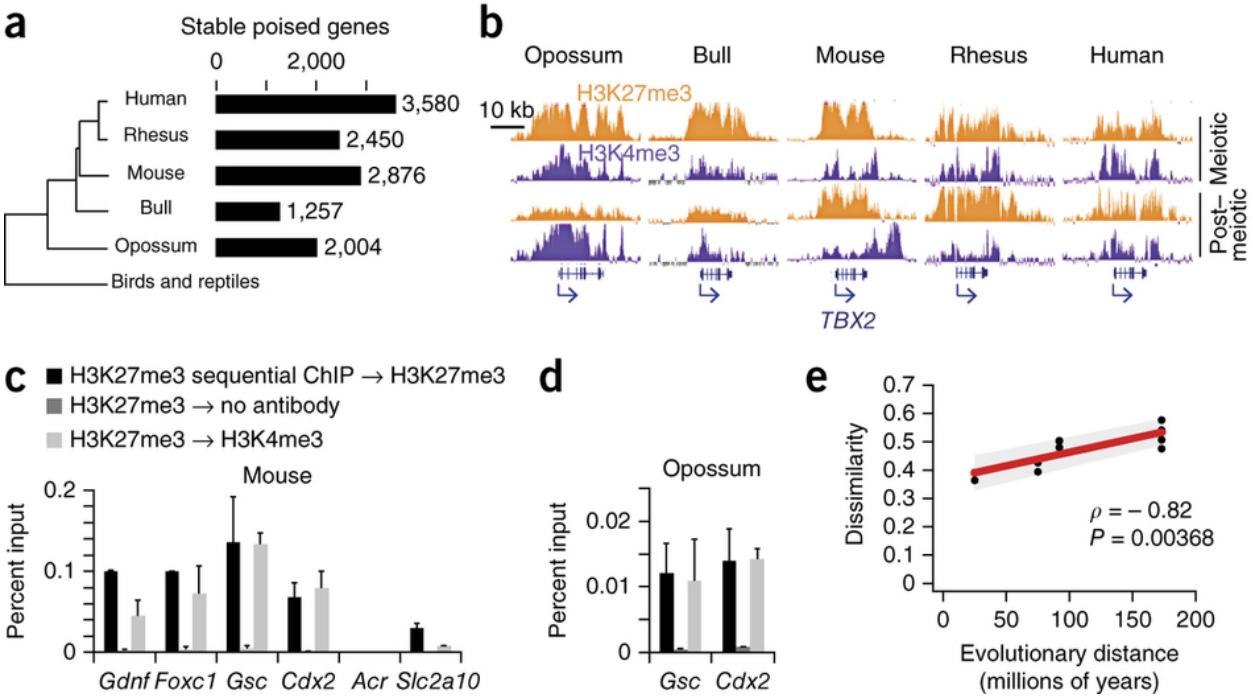

Figure 2. The poised chromatin state in the mammalian germ line. **a**, Numbers of stable poised genes (called as poised in both pachytene spermatocytes and round spermatids) in each species. **b**, Input-subtracted gene tracks showing H3K4me3 and H3K27me3 signal in all five species at one representative poised gene, *TBX2*. kb, kilobases. **c**, Quantitative PCR (qPCR) data from sequential ChIP experiments at four representative poised promoters, one H3K4me3-only promoter (*Acr*), and one H3K27me3-only promoter (*Slc2a10*) in mouse round spermatids. Bar height shows the mean and error bars represent s.d. for three biological replicates (*Gdnf* and

Foxc1) or three technical replicates (*Gsc*, *Cdx2*, *Acr*, *Slc2a10*). Browser tracks corresponding to the assayed regions are shown in Supplementary Figure 7. **d**, qPCR data from sequential ChIP experiments at two representative poised promoters in opossum round spermatids. Bar height shows the mean and error bars represent s.d. for three technical replicates. Browser tracks corresponding to the assayed regions are shown in Supplementary Figure 7. **e**, Dissimilarity in poised gene sets (fraction of poised genes not shared) between pairs of species as a function of divergence time. Grey shading indicates 95% confidence interval. ρ, Spearman's correlation coefficient.

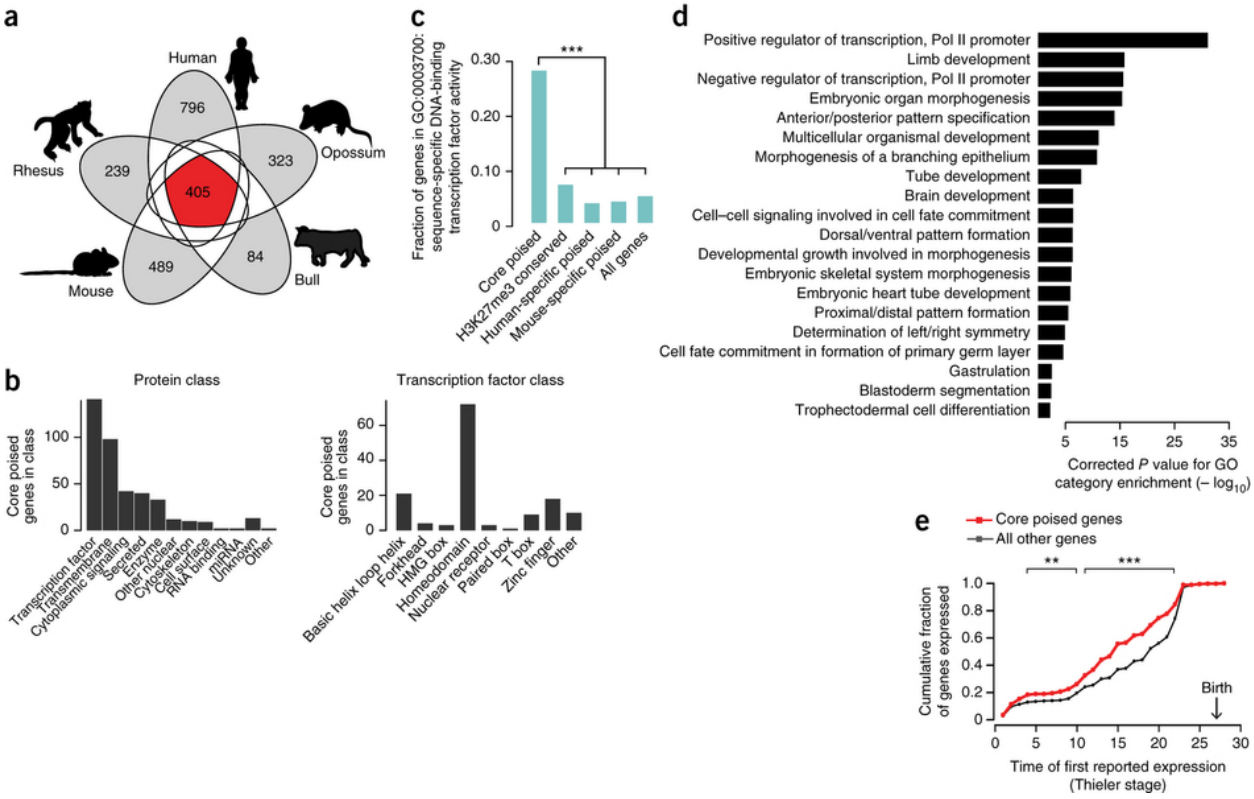

Figure 3. Core poised genes are conserved regulators of tissue patterning. **a**, Overlap between poised gene sets in five mammalian species. The core poised gene set is highlighted in red and differentially poised gene sets in grey. **b**, Classification of molecular function and transcription factor family for the 405 genes poised in all five mammalian species. **c**, Fraction of each gene set included in the GO category "sequence specific DNA binding transcription factor

activity" (GO:0003700). ***p < 0.001 (Fisher's exact test). **d**, Selected enriched GO categories for the core poised genes. p-values were calculated using a hypergeometric test and corrected for multiple testing (Methods). See Supplementary Table 5 for a complete list. **e**, Cumulative fraction of core poised genes expressed throughout mouse embryogenesis, compared to all genes with five-way orthologs. **p<0.01, ***p < 0.001 (Fisher's exact test).

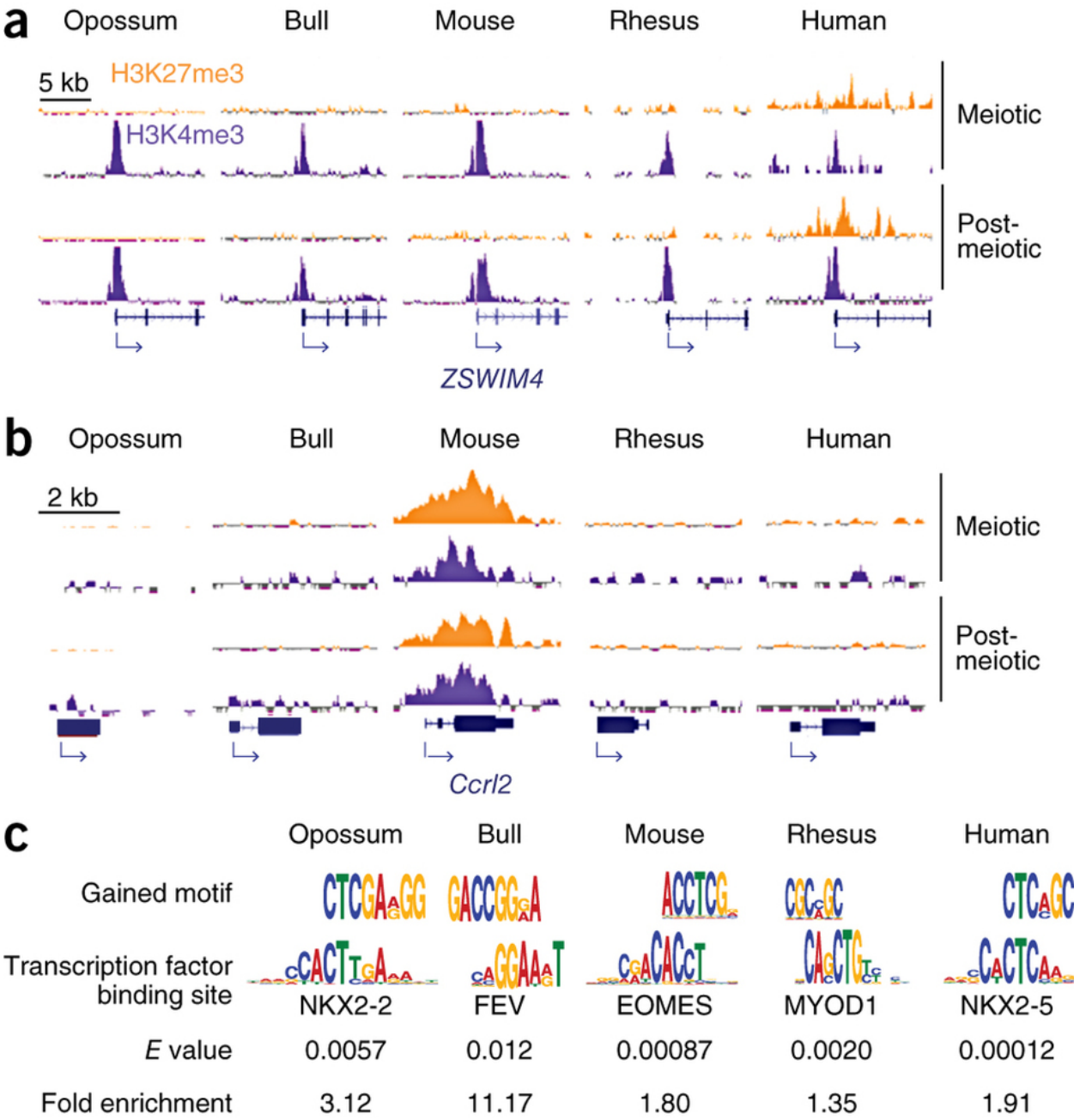

Figure 4. Gain of poising at genes with species-specific developmental roles. **a**, **b**, Input-subtracted gene tracks showing H3K4me3 and H3K27me3 signal in all five species at (**a**) the human-specific poised gene *ZSWIM4,* which is expressed in human but not mouse or bull placenta and (**b**) the mouse-specific poised gene *Ccrl2*, which is expressed in mouse but not human or bull placenta. kb, kilobases. **c**, Representative gained motifs in the promoters of differentially poised genes in each species (top) aligned with binding motifs for transcription factors encoded by core poised genes. The core transcription factor corresponding to the binding site is indicated below each motif, along with E-values (p-value for motif enrichment times number of motifs tested) and fold enrichment of the gained motif compared to orthologous sequences.

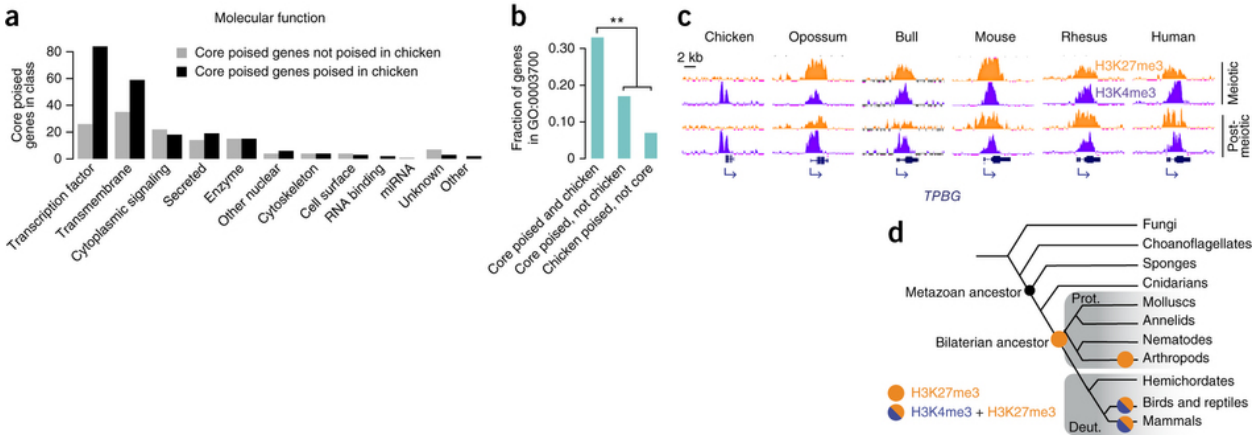

Figure 5. Conservation of poising in the metazoan germ line. **a**, Classification of molecular function for the 347 genes poised in germ cells of all five mammalian species that have orthologs in the chicken genome. Black bars represent the 215 genes poised in all five mammals and chicken; grey bars represent the 132 genes poised in all five mammals but not chicken. **b**, Fraction of each gene set that is included in the GO category "sequence specific DNA binding transcription factor activity" (GO:0003700). **p < 0.01 (Fisher's exact test). **c**, Input-subtracted gene tracks showing H3K4me3 and H3K27me3 signal in all five mammalian species and

chicken at a gene poised specifically in mammals, *TPBG*. kb, kilobases. **d**, Metazoan cladogram showing lineages with evidence for H3K4me3 and H3K27me3 (purple and orange circles) or H3K27me3 only (orange circles) at orthologs of the core poised genes in germ cells. When no circle is shown, appropriate data is not available. Shaded grey boxes indicate protostome (prot.) and deuterostome (deut.) lineages.

Online Methods

Human subjects

These studies were approved by the Massachusetts Institute of Technology's Committee on the Use of Humans as Experimental Subjects. Informed consent was obtained from all subjects.

Human sample collection and sorting

Human testis samples were obtained from adult male patients undergoing vasectomy reversals at the Infertility Clinic of St. Louis. All men whose tissue was used in this study had a prior history of fertility demonstrated by at least one living child. Epididymal sperm quality and abundance proximal to the vasectomy site was assessed at the time of biopsy, and abundant, motile, morphologically normal sperm were confirmed for each patient. Testis biopsy samples were minced, dissociated using collagenase and trypsin, and then filtered to obtain a single-cell suspension as described[59]. Pachytene spermatocyte and round spermatid fractions were collected by StaPut[59-61], and pooled fractions were counted on a hemocytometer. Purity was >95% for each sample, as assessed by counts of 100 cells from each fraction under phase optics. Cells were washed once in PBS and then split into two aliquots. One aliquot (for ChIP) was fixed in 1% formaldehyde for 8 minutes at room temperature and then quenched with 2.5M glycine for 5 minutes at room temperature, while the second (for RNA) was kept on ice during this time. Both fixed and unfixed aliquots were snap frozen in liquid nitrogen, then stored at -80C.

Non-human sample collection and sorting

Testes from rhesus monkeys were obtained from adult male animals undergoing necropsy for other purposes at the Texas Biomedical Research Institute (TBRI). The necropsy procedure was approved in advance by the TBRI Institutional Animal Care and Use Committee (IACUC). Procedures involving mice were approved in advance by the IACUC of the University of Texas at San Antonio. Testes were isolated from adult male CD1 mice (Charles River Laboratories), and tissue from several mice was pooled before cell separation. Testes from grey short-tailed opossums (*Monodelphis domestica*) were obtained from adult male animals culled from a colony maintained at the TBRI. Euthanasia of these animals was also approved by the TBRI IACUC. Testes from a bull and three roosters were obtained as abattoir material that would otherwise have been discarded. Tissue from the three roosters was pooled before cell separation. In each case, populations of pachytene spermatocytes and round spermatids were recovered using a StaPut gradient as described[17,59,62-65] and prepared for ChIP or RNA-Seq as described above and elsewhere[17]. Purity was assessed by counting 100 cells from each fraction under phase optics. Purity was 89-90% for *Monodelphis* samples, and >90% for samples from the other species.

RNA isolation

Unfixed aliquots of sorted cells were thawed on ice, washed once in cold PBS, resuspended in 350 ul RLT Plus buffer from the RNEasy Mini kit (Qiagen #74134) and then disrupted by drawing up and down five times in a 26G insulin needle and syringe. Genomic DNA was removed using gDNA eliminator columns supplied with the kit. The remainder of the RNA isolation was performed using the RNEasy Mini kit according to the manufacturer's instructions.

Samples were processed in batches of 2-6 in the order of collection with no blinding. All biological replicates were processed in separate batches from each other.

Chromatin immunoprecipitation

For ChIP-seq, between 5 x 10^4 and 5 x 10^6 cells were used as starting material, depending on the number obtained from sample isolation and sorting. Pachytene spermatocytes and round spermatids were treated identically. For human samples, fixed cells frozen in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8]) were thawed on ice. For non-human samples, fixed cell pellets were thawed on ice, then washed once in cold PBS and resuspended in 100 ul lysis buffer. Once in lysis buffer, cells were incubated on ice 5 minutes. 200 ul ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8], 167 mM NaCl) was then added to each sample. Samples were then sonicated in aliquots of 150 ul in 0.5 ml Eppendorf tubes at 4C using a BioRuptor (Diagenode) for 35 cycles on High setting, 30 seconds on/30 seconds off. Aliquots of the same sample were then re-pooled and spun down at 12,000 xg for 5 minutes, and the chromatin supernatant moved to a fresh tube. Chromatin from each sample was then split into two separate tubes (150 ul in each), and 700 ul dilution buffer, 50 ul lysis buffer, and 100 proteinase inhibitor cocktail (Complete Mini tablets, Roche #11836153001) were added to each tube. 50 ul of each sample was set aside as input. The remainder of the ChIP was performed as previously described[17], using 0.5 ug of antibody to H3K4me3 (abcam #ab8580) or 1.0 ug of antibody to H3K27me3 (abcam #ab6002). Samples were processed in batches of 2-4 in the order of collection with no blinding; pachytene spermatocytes and round spermatids from a given sample were processed side by side, and all biological replicates were processed in separate batches from each other.

Sequential chromatin immunoprecipitation

Sequential ChIP was performed using the Re-ChIP-IT kit from Active Motif (#53016) according to the manufacturer's instructions. 5 x 10^7 mouse round spermatids or 2 x 10^7 opossum round spermatids were used as starting material for each experiment. The first ChIP was performed with 2 ug of antibody to H3K27me3 (abcam, #ab6002). The second ChIP was performed with either (1) 1.5 ug of antibody to H3K4me3 (abcam, #ab8580), (2) 2.5 ul of antibody to H3K27me3 (Millipore #07449) as a positive control, or (3) 2 ul of water as a negative control.

Quantitative PCR

All primer sequences are listed in Supplementary Table 10. Quantitative PCR (qPCR) was performed on an Applied Biosystems 7500 Fast Real-Time PCR instrument using Applied Biosystems Power SYBR Green PCR Master Mix with the following cycling conditions:

1. 50° 0:20
2. 95° 10:00
3. 95° 0:15
4. 60° 1:00
5. 70° 0:30
6. Go to step 3 x39

For targets with biological replicates (*Gdnf* and *Foxc1*), we observed variable ChIP efficiency between experiments done on different days. To compare the relative percent input values between experimental and control conditions across biological replicates, all values from a given

experiment were proportionately scaled such that the value of the positive control condition (H3K27me3 → H3K27me3) was 0.1% input.

Antibodies

Anti-H3K4me3 (rabbit polyclonal, Abcam #8580) was used for ChIP-seq in all species and for sequential ChIP in mouse and opossum. This antibody has been validated for ChIP-seq applications in human (Histone Modification Antibody Validation Database)[66], rhesus macaque[67], and mouse[68], as well as non-mammalian species including *Drosophila*[66]. Anti-H3K27me3 (mouse monoclonal, Abcam #6002) was used for ChIP-seq in all species and for sequential ChIP in mouse and opossum. This antibody has been validated for ChIP-seq applications in human[69], mouse[70], and chicken[71], as well as non-mammalian species including *Drosophila*[66] and *C. elegans*[66]. Anti-H3K27me3 (rabbit polyclonal, Millipore #07449) was used for sequential ChIP in mouse and opossum. This antibody has been validated for ChIP applications in mouse[68], human[66], and *Drosophila*[72].

Illumina library preparation and sequencing

RNA libraries were prepared using an Apollo 324 library prep instrument with supplied reagents (Integenx) for non-human samples, and using a SMARTer stranded RNA prep kit (Clontech) for human samples, according to the manufacturer's instructions. ChIP libraries were prepared using a TruSeq ChIP sample prep kit (Illumina), according to the manufacturer's instructions, except that size selection was performed after (instead of before) PCR amplification. Data from mouse replicate #1 has been previously published[17]; for this sample, ChIP- and RNA-seq libraries were sequenced on an Illumina GAII with 36-base-pair single-end reads. All other

libraries were sequenced on an Illumina HiSeq2500, with 40-base-pair single-end reads for ChIP libraries and 100-base-pair or 40-base-pair paired end reads for RNA-seq libraries (Table S1).

Sequence alignment

We filtered all datasets for read quality using FASTX-toolkit and assessed library quality using FastQC. We aligned ChIP-seq libraries to a species-appropriate genome build (hg19, rheMac2, mm10, bosTau7, monDom5, or galGal4) using Bowtie v1.1.1[73] (Table S1). For ChIP-seq data, we called peaks at a threshold of $p < 10^{-6}$ using MACS v1.4[74]; the number and locations of peaks were used to evaluate the quality of the dataset, but were not used in our analysis. For all datasets, peak numbers were within the expected range for the appropriate histone modification (H3K4me3 or H3K27me3); variation in peak numbers within this range did not strongly affect poised gene calls. For RNA-seq data, we aligned libraries using Tophat v2.0.11[75] with Ensembl[76] (release 75) transcripts as a reference (-G flag). The default genome assemblies included in Ensembl release 75 matched those used for alignment for all species except bull. For bull, Ensembl coordinates (for bosTau6) were mapped to the bosTau7 assembly using CrossMap[77].

Due to the small cell numbers of many of the sorted cell populations, both ChIP and RNA-seq libraries tended to have high duplication levels (Table S1). We treated duplicate reads conservatively for both ChIP- and RNA-seq data. For ChIP-seq data, where a minimum count threshold was used for poised gene calls, we retained only one duplicate for analysis. For RNA-seq data, where a maximum threshold was used, we retained a maximum of 20 (Tophat default) duplicate reads.

691

692 **Poised gene calls**

693 For ChIP data, we counted total reads in the 4-kb interval surrounding each transcript TSS
694 (Ensembl build 75) using htseq-count[78] with the intersection-nonempty option. The 4-kb interval
695 (2-kb upstream and 2-kb downstream of the TSS) is standard for analysis of promoter-associated
696 histone modifications. For each dataset, total ChIP or input reads in each interval were
697 normalized to reads per million, and the normalized input count was subtracted from the
698 normalized ChIP count in each interval to get a final ChIP signal. For RNA, we obtained FPKM
699 values using Cufflinks v2.2.1[79], with Ensembl release 75 transcripts as a reference (-G option).
700 Transcript values for both ChIP and RNA-seq data were summed to get a single H3K4me3,
701 H3K27me3, and expression value for each gene. We conducted simulations in which we varied
702 ChIP and expression thresholds and evaluated the numbers of poised genes called in each
703 species, and selected thresholds that included the maximum number of poised genes while
704 remaining robust to small changes in threshold value (Supplementary Figs. 5 and 6,
705 Supplementary Data). We set thresholds of ≥ 0.5 input-subtracted reads per million for
706 H3K4me3 signal, ≥ 0.5 input-subtracted reads per million for H3K27me3 signal, and ≤ 5 FPKM
707 for expression. For each sample, a gene had to meet thresholds for H3K4me3, H3K27me3, and
708 expression in both pachytene spermatocytes and round spermatids (six data points total) to be
709 considered stably "poised". For species with two biological replicates (rhesus, mouse, and
710 opossum), we used mean ChIP signal and FPKM values to call poised genes for that species; this
711 approach yielded similar gene lists to either the union or intersection of the two replicates, but
712 was more robust to changes in threshold. For species with three biological replicates (human),
713 we included genes called as poised in at least two out of the three individual replicates. We note

that these criteria are expected to result in greater sensitivity of poised gene calls for species with more replicates, since use of more replicates allows inclusion of genes that may fail to meet one of the six thresholds in a single replicate. We do observe the fewest poised gene calls in bull (one replicate) and the most in human (three replicates). These differences in sensitivity may result in a subset of false positives and negatives in lists of genes called as differentially poised between species. The list of conserved H3K27me3-only genes (Figures 3c, S8b, Supplementary Table 5) was defined as the set of genes that met H3K27me3 and expression thresholds in all five mammalian species, but met the H3K4me3 threshold in fewer than four out of five.

Orthologous gene sets

We required that a gene have orthologs in all five mammalian species in order to be included in our analysis. Since there is no strong *a priori* expectation that gene duplication would have a specific effect (loss, gain, or retention) on chromatin state surrounding the TSS, we reasoned that exclusion of genes with 1-to-many relationships could result in loss of biologically meaningful information and might introduce bias by excluding a non-random set of genes from the analysis. We therefore included genes with either 1-to-1 or 1-to-many orthology relationships among the five species. We used the BioMart database[80] with Ensembl release 75 to find 1-to-1 and 1-to-many orthologs among the five species. In total, we identified 14362 orthology groups containing at least one gene from each species. 12104 of these involved only 1-to-1 orthology relationships across all five species; we used this number to calculate p-values for the significance of five-way overlaps (see below). The 14362 orthology groups included a total of 15492 human, 15904 rhesus, 16253 mouse, 15650 bovine, and 15966 opossum genes, which together comprised the total gene set considered in our downstream analysis. When determining

sets of overlapping poised genes, an orthology group was counted as overlapping if at least one gene belonging to the group was poised in each species (Supplementary Data and Supplementary Code).

Statistics

Sample inclusion criteria. Testis samples were excluded from the study if any morphological abnormality was observed in the intact tissue. For any ChIP-seq or RNA-seq dataset with $<10^6$ unique (nonduplicate) reads aligning uniquely to the genome, the associated biological sample and all datasets derived from it were excluded from analysis. These criteria were established prior to beginning the study. When possible, at least two biological replicates were obtained to allow for individual variation. For human data, three biological replicates were used in the final analysis to account for greater variability in genetic background compared to non-human species.

Statistical tests. Categorical data comparisons were evaluated using hypergeometric tests (Fisher's exact test). For comparisons of continuously distributed data, we used a two-sided Welch t-test for statistical comparison, which is robust to non-normal distributions at large sample sizes and also accounts for unequal variance between groups. We assessed variance using the Brown-Forsythe test. For ranked-list comparisons, we used a one-sided Mann-Whitney U test.

Gene set overlaps. Overlaps between multiple (>2) gene sets were computed using the overLapper function from the systemPipeR package in R[81]. Statistical significance of five-way overlap was derived using the formula $p < \binom{N}{m}\left[\frac{\binom{N-m}{n-m}}{\binom{N}{n}}\right]^5$, where N = total number of genes with orthologs in all five species, n = largest number of poised genes called in any single species (within the set of genes with orthologs in all five species), and m = number of genes called as

poised in all five species. Using our gene set, N=14362, n=3580, m=405, and $p<10^{-300}$ in this calculation. However, we note that core poised genes are enriched for genes with true one-to-one orthologs, meaning that the groups being compared are not completely independent as the formula assumes. To account for this bias, we re-calculated the overlap between gene sets, including only 1-to-1 orthologs in the analysis. With 1-to-1 orthologs only, N=12104, n=3361, m=401, and $p<10^{-280}$. We report this p-value as a conservative estimate of significance for the five-way overlap.

Gene ontology enrichment

GO enrichments were evaluated using the GOStats package[82] in R. p-values were adjusted both by conditioning out child categories and by subsequent correction for multiple testing using the Benjamini-Hochberg method.

Clustering and divergence estimates

We generated a distance matrix for expression datasets based on FPKM, and for ChIP datasets based on normalized counts around the promoter regions (Supplementary Data), using 1-ρ (Spearman's correlation) as a dissimilarity metric[49]. Clustering was performed and dendrograms generated using the 'cluster' package[83] in R. Analysis of gene expression and promoter chromatin divergence was carried out using dissimilarity scores between each species pair, and data from each cell type was fit to a linear model (Supplementary Code).

Principal component analysis

We used the 'PCA' function from the FactoMineR package[84] in R for principal component analysis, with data scaled to unit value. Input data was the same processed data (normalized H3K4me3 signal, normalized H3K27me3 signal, or FPKM) as was used for calling poised genes (Supplementary Data).

Somatic tissue expression

The Mouse Genome Informatics (MGI) Gene Expression Database[21] was used to determine stages of gene expression for mouse poised genes. Only wild type samples from the database were used in the analysis. At the time of our study, the database included a total of 13837 genes; 9884 genes in the database had orthologs in all five mammalian species. The numbers of genes in the database with orthologs in all five species (359 core poised genes and 9525 other genes) were used as denominators in calculating the fraction of genes expressed at each stage.

Embryonic lethality

We identified alleles associated with embryonic lethality by searching the MGI database using the Phenotypes, Alleles, and Disease Models query[31] for "embryonic lethality" (phenotype ID MP:0008762) and filtering for null/knockout alleles. This search identified 2812 alleles, corresponding to 1881 genes; 1570 of these genes had orthologs in all five mammalian species.

Transcription factor class enrichment

Transcription factor classes (Fig. 3b, Supplementary Fig. 8c) were assigned according to Wingender et al., 2013[85].

Motif analysis

We identified motifs enriched in promoters of species-specific poised genes in two steps. In the first step, we used DREME[86] with default settings to detect motifs enriched in each set of differentially poised promoters (+/- 1kb from the transcription start site) compared to orthologous promoter regions of the other four species, with a threshold of E<0.05. In the second step, to control for the biases introduced from comparing different species, we used AME[87] to scan 100 random, equally-sized sets of orthologous promoters for enrichment of the motifs detected in step (1) in the species in which they were first detected compared to the other four species. The fraction of random promoter sets demonstrating enrichment at E<0.05 constituted a raw p-value; these values were adjusted for multiple comparisons using the Benjamini-Hochberg approach to get a false discovery rate for each enriched motif. We considered only motifs with FDR < 0.10 in our subsequent analysis. To match enriched motifs with binding sites for known transcription factors, we used Tomtom[88] (conditions: -thresh 10 -evalue -dist ed), and pulled motifs from the JASPAR Core vertebrates[89] (205 total motifs) and Uniprobe mouse[90-92] (386 total motifs) databases.

Drosophila Polycomb data

Drosophila ChIP-chip data was taken from El-Sharnouby et al, 2013[50]. Using tiled enrichment values generated by the authors, we calculated average Polycomb enrichment within 1 kilobase upstream or downstream of each *Drosophila* TSS and assigned these values to the associated gene. We designated genes in the top 25% of Polycomb signal as “high Polycomb”, and genes in the bottom 25% as “low Polycomb”.

Code availability

Custom R scripts used in our analyses are included as Supplementary material (SupplementaryDataSet1.zip).

Supplementary references

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Competing financial interests

The authors have no competing financial interests.