Genome-wide identification of regulatory elements in Sertoli cells

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ABSTRACT

A current goal of molecular biology is to identify transcriptional networks that regulate cell differentiation. However, identifying functional gene regulatory elements has been challenging in the context of developing tissues where material is limited and cell types are mixed. To identify regulatory sites during sex determination, we subjected Sertoli cells from mouse fetal testes to DNaseI-seq and ChIP-seq for H3K27ac. DNaseI-seq identified putative regulatory sites around genes enriched in Sertoli and pregranulosa cells; however, active enhancers marked by H3K27ac were enriched proximal to only Sertoli-enriched genes. Sequence analysis identified putative binding sites of known and novel transcription factors likely controlling Sertoli cell differentiation. As a validation of this approach, we identified a novel Sertoli cell enhancer upstream of *Wt1*, and used it to drive expression of a transgenic reporter in Sertoli cells. This work furthers our understanding of the complex genetic network that underlies sex determination and identifies regions that potentially harbor non-coding mutations underlying disorders of sexual development.

KEY WORDS: Genome-wide enhancer profiling, Sex determination, Sertoli cell development, Mouse

INTRODUCTION

During development, multipotent progenitor cell commitment to one cell fate versus another is driven by complex gene networks controlled by diverse regulatory factors, including transcription factors (TFs), chromatin remodelers and non-coding RNAs (ncRNAs). Although identification of the cohorts of genes that drive cell fate determination is an important focus of developmental studies, it is equally important to identify the regulatory sites through which these factors operate. Mutations of regulatory sites in enhancers and promoters that alter gene expression have been discovered serendipitously, but until recently, an unbiased genome-wide approach to identify regulatory elements in specific fetal cell types remained difficult. Thus, it has not been clear how the accessibility of chromatin sites and the availability of TFs act together to control cell fate decisions during fetal development from a pluripotent to a committed fate.

The developing gonad is a powerful model system for understanding the gene regulatory mechanisms that drive cell fate determination. The process of sex determination occurs in gonad progenitor cells whose differentiation controls the sexual fate of the organism. Although genetic sex (XY/XX) is established upon fertilization, these innate chromosomal differences do not produce phenotypic differences in male or female early embryos (Eggers and Sinclair, 2012). Instead, sexual dimorphism is initiated in the early gonad supporting cells, a single cell lineage that differentiates into Sertoli (XY) (Sekido et al., 2004) or pregranulosa (XX) cells (Albrecht and Eicher, 2001; Mork et al., 2012). Prior to sex determination, XX and XY supporting cells are bipotential and express nearly all genes at similar levels, with minor differences limited to genes on the X and Y chromosomes (Nef et al., 2003; Munger et al., 2013).

In the mouse, male sex determination is initiated around embryonic day 11.5 (E11.5) by expression of the Y-linked gene *Sry*, which diverts XY supporting cells to the Sertoli cell fate (Fig. 1A). *Sry* is transiently expressed and many SRY-binding sites are subsequently bound by its downstream target SOX9 (Li et al., 2014). SRY and SOX9 control the differentiation of Sertoli cells by triggering a dramatic transcriptional reprogramming of bipotential progenitor cells within just 24 h, leading to the upregulation of over 200 genes important for Sertoli cell development and downregulation of ~100 pregranulosa cell-expressed genes that were expressed at the bipotential stage (Munger et al., 2013). In the absence of *Sry*, XX supporting cells differentiate to pregranulosa cells, but in contrast to the dramatic transcriptional changes that accompany Sertoli cell specification, pregranulosa cell differentiation proceeds with fewer transcriptional changes consistent with the finding that undifferentiated XX and XY supporting cells are initially transcriptionally biased toward ovarian fate (Jameson et al., 2012b).

Although *SRY* acts as a pivotal switch in the sex-determining pathway, mutations in *SRY* account for only ~10-15%, of XY disorders of sex development (DSDs) (Cameron and Sinclair, 1997). Over 35 other genes have been identified that contribute to DSDs (Arboleda and Vilain, 2011; Arboleda et al., 2013), suggesting that a complex genetic network controls fate commitment in the early gonad. It is evident from human DSD cases where gene dose is altered by duplications or haploinsufficiencies (Foster et al., 1994; Muscatelli et al., 1994; Zanaria et al., 1994; Jordan et al., 2001) that perturbations to the level or timing of individual genes within this network can lead to sex reversals. In the C57BL/6J inbred mouse strain, the altered expression of several sex-determining genes causes unique strain-dependent sex-reversal phenotypes in response to specific mutations (Colvin et al., 2001; Bouma et al., 2005; Kim et al., 2007b; Munger et al., 2009, 2013; Correa et al., 2012; Warr et al., 2012). These studies point to the importance of precise spatial and temporal gene

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¶Deceased: this paper is dedicated to Danielle's memory

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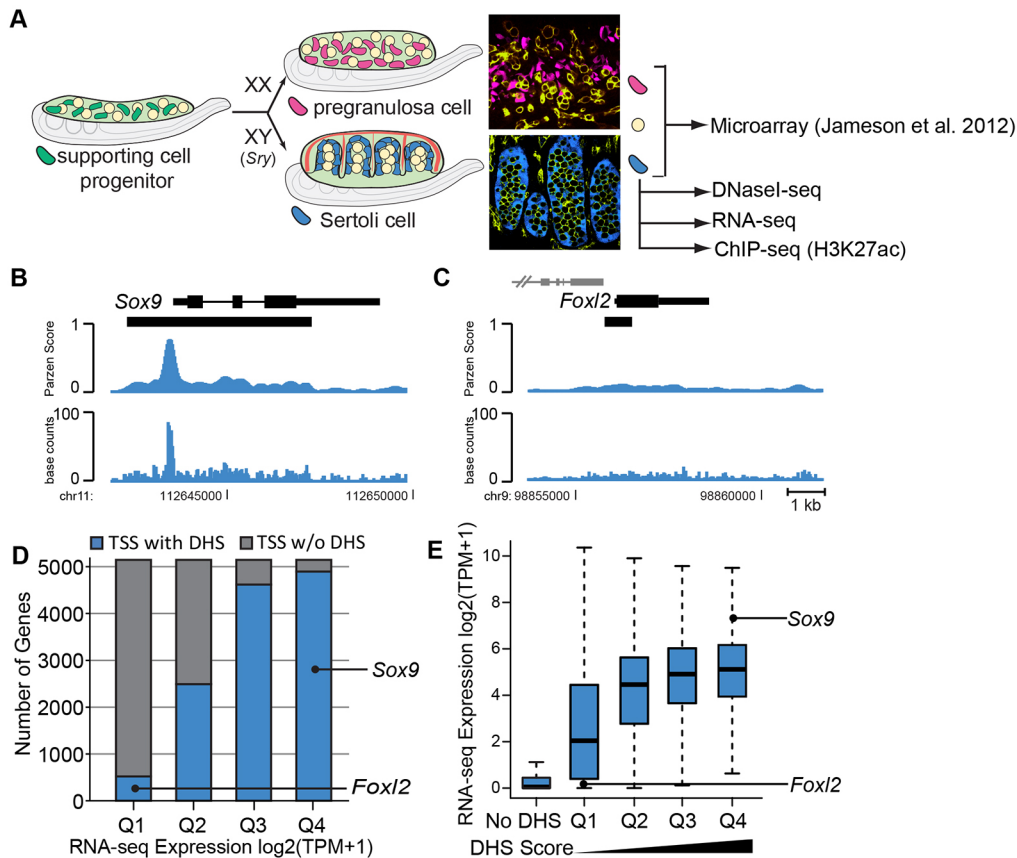D.M.M., 0000-0002-4303-1187; B.C., 0000-0002-6587-0969

Fig. 1. DNaseI-seq and RNA-seq analysis of Sertoli cells. (A) Overview of sex determination. At the bipotential stage (E11.5), XX and XY gonads are indistinguishable and contain a mixture of somatic and germ cells (yellow). The supporting cell lineage (green) gives rise to Sertoli cells of the testis (XY; blue) or pregranulosa cells of the ovary (XX; pink) upon sex determination. Confocal images show a whole-mount immunostained E13.5 ovary (top) and testis (bottom). XX pregranulosa cells express the *Sry-eGFP* transgene (Albrecht and Eicher, 2001) (pink), XY Sertoli cells express the *Sox9-CFP* transgene (blue; germ cells and vasculature are yellow/green). Microarray data were previously collected from FACS-isolated E13.5 pregranulosa cells, Sertoli cells and germ cells (Jameson et al., 2012b). DNaseI-seq was performed on FACS-isolated E13.5 and E15.5 Sertoli cells. RNA-seq was performed on E15.5 Sertoli cells. (B,C) DNaseI-seq data identified a strong DHS at (B) the *Sox9* promoter (expressed in Sertoli cells) and a weaker DHS at (C) the *Foxl2* promoter (repressed in Sertoli cells). Only peaks overlapping the TSS are shown and gene names are positioned adjacent to the TSS. Nearby genes are indicated in gray. Black bars under the gene indicate DHSs. The top track indicates the Parzen score, or DHS score, while the bottom track shows the smoothed base counts. (D,E) Comparative analysis of E15.5 DNaseI-seq and RNA-seq data. (D) RNA-seq data [log base 2 (transcripts per million (TPM+1))] from E15.5 Sertoli cells was divided into quartiles based on expression values [Q1, $\log_2$(TPM+1)=0-0.1; Q2, $\log_2$(TPM+1)=0.1-2.34; Q2, $\log_2$(TPM+1)=2.34-5.02; Q2, $\log_2$(TPM+1)=5.02-12.74]. The chart indicates the number of genes within each quartile that had an overlapping DHS (blue) or did not have an overlapping DHS (gray) at the TSS. (E) Genes with a DHS overlapping the TSS were divided into quartiles based on DHS scores (Q1-Q4, low to high DHS scores). The distribution of expression values for each group is shown as a boxplot (excluding outliers).

regulation. However, our lack of knowledge regarding the locations of crucial gene-regulatory sites limits our ability to study the regulatory mechanisms that control gene expression in Sertoli cells.

To identify putative regulatory sites in Sertoli cells, we performed DNaseI-seq on purified Sertoli cells from fetal testes just after sex determination. Comparison with DNaseI-seq data from a variety of other mouse tissues and cell types revealed thousands of novel Sertoli cell-specific putative regulatory elements, which are enriched near genes expressed in Sertoli cells, as well as genes that are silent in Sertoli cells, but expressed in the alternate pregranulosa cell lineage. This suggests that DNaseI-seq identified sites involved in the activation and repression of genes required for Sertoli cell fate commitment. Using ChIP-seq for H3K27ac, we located active enhancer elements specifically enriched near genes expressed in Sertoli cells, and used this data to identify TF motifs that distinguish active from inactive enhancers. Finally, we validated the enhancer activity of an active regulatory element located 50 kb upstream of *Wt1*, which encodes a TF essential for early gonad development. This data will enable future experiments to characterize the regulatory

network driving Sertoli cell development and may aid in the identification of non-coding human mutations underlying unexplained cases of DSDs, the majority of which cannot be attributed to mutation within the coding region of a known sex-determining gene (Arboleda and Vilain, 2011; Arboleda et al., 2013).

RESULTS
Adapting DNase1-seq to developmentally limited cell populations

We conducted a proof-of-principle experiment to establish the efficacy of DNaseI-seq in identifying regulatory elements in Sertoli cells that had been purified by fluorescence-activated cell sorting (FACS) followed by long-term storage at −80°C. E15.5 Sertoli cells were isolated by FACS based on the Sertoli cell-specific expression of the *Sox9-CFP* transgene (Fig. 1A). Significantly reducing the starting material required, we found that 5×10^6 cells routinely yielded enough chromatin to perform the DNaseI-seq assay.

DNaseI-seq identified over 81,000 DNaseI hypersensitive sites (DHSs) in E15.5 Sertoli cells. Using previously published methods

(Song et al., 2011), DHSs were determined to be in gene promoters (23%), intergenic (31%) and genic regions (exons 10%, introns 36%), and displayed a median length of 330 bp (Fig. S1). Over 13,000 genes (~60%) had a DHS peak overlapping a transcriptional start site (TSS), whereas over 9000 TSSs (~40%) lacked a DHS peak. Two loci representing a gene expressed in Sertoli cells (*Sox9*) and a granulosa-specific gene (*Foxl2*) displayed DNase signals of differing magnitudes at their TSS (Fig. 1B,C), suggesting that the DHS signal at the TSS may correlate with gene expression levels. To examine this possibility, RNA-seq was performed on E15.5 Sertoli cells (Table S1). Consistent with prior studies (Boyle et al., 2008a), the presence and magnitude of a DHS peak at the promoter was associated with increased gene expression values (Fig. 1D,E).

Further validating the quality of our data, we identified the only known distal enhancer for a Sertoli cell-expressed gene: *Sox9*. Although the *Sox9* enhancer TES is likely over-represented in our data set due to the multi-copy nature of transgene insertions in the *Sox9 (Tes)-CFP* line, our DHS data identifies only the smaller functional element, TESCO (Sekido and Lovell-Badge, 2008), as having putative enhancer activity (Fig. 2A).

Identification of DHSs important for gene regulation in Sertoli cells

Given the success of this experiment, we performed DNaseI-seq on E13.5 Sertoli cells, a stage of development for which we have extensive expression data (Nef et al., 2005; Bouma et al., 2007a, Jameson et al., 2012b) (Table S2). Our E13.5 DNaseI-seq data showed similar properties to the data from E15.5 Sertoli cells; ~71% of the DHSs were shared between both data sets and had nearly identical genomic distributions (Fig. S2A,B).

To identify the DHSs most relevant for gene expression during testis development, we conducted DNaseI-seq on six other mouse samples [primary skin fibroblasts, embryonic stem cells (ESCs), adult kidney, liver, heart and brain]. To identify putative enhancers important for gene expression in Sertoli cells, we used the six other somatic cell types to classify E13.5 and E15.5 Sertoli cell DHSs as unique (present in only Sertoli cells), shared (in Sertoli cells and in

one to five additional cell types) or common (in all cell types). For example, at the *Sox9* promoter, all cell types had a DHS (common DHS); however, the TES enhancer was found only in the E13.5 and E15.5 Sertoli cell datasets (unique DHS) (Fig. 2A). Twenty-seven percent (21,743) of all E15.5 Sertoli cell DHSs and 25% (21,863) of E13.5 Sertoli cell DHSs were common, with promoters accounting for over half of the common sites (Fig. 2B; Fig. S2C). CTCF binding sites are highly conserved across different cell types (Kim et al., 2007a; Xi et al., 2007; Song et al., 2011) and, consistent with this, CTCF-binding sites found in eight other cell types (Shen et al., 2012) were enriched in the common DHSs (Fig. 2C). In fact, of the common sites not overlapping a promoter, ~75% overlapped a CTCF binding site. The remaining DHSs were unique (E15.5: 29%; 23,548, E13.5: 32%, 28,133) to the Sertoli cell lineage or shared (E15.5: 44%; 36,176, E13.5: 43%, 38,111). A high proportion of unique (85%) and shared (73%) sites localized to intergenic or intronic regions (Fig. 2B; Fig. S2B), and are likely to be important regulators of gene expression in Sertoli cells.

Sertoli cell DHSs associate with Sertoli and pregranulosa cell-expressed genes, whereas active enhancers are enriched near Sertoli cell genes

The DHSs identified by DNaseI-seq correlate with several genomic features, including promoters, enhancers, repressors and insulators (Heintzman et al., 2007, 2009; Boyle et al., 2008a). To focus on regions of the genome driving Sertoli cell-specific gene expression, we conducted chromatin profiling for H3K27ac, a strong predictor of active enhancer activity (Creyghton et al., 2010; Rada-Iglesias et al., 2011). H3K27ac ChIP-seq on E13.5 Sertoli cells identified >28,000 H3K27ac peaks, ~70% of which overlapped a DHS in our E13.5 Sertoli cell dataset (Fig. 3A, Fig. S4).

We predicted that active enhancers would be enriched around Sertoli cell-expressed genes. To test this, the enrichment of DHSs, or H3K27ac-positive DHSs, was examined around Sertoli cell-expressed genes and compared against genes expressed in pregranulosa and germ cells (Jameson et al., 2012b). Using a nearest-neighbor approach (McLean et al., 2010), we assigned
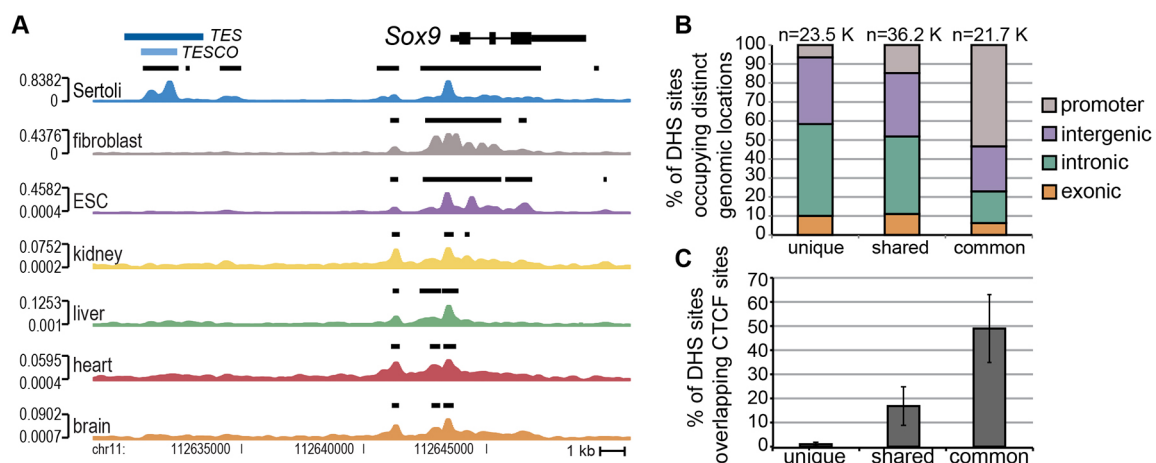

Fig. 2. Identification of DHSs unique to the Sertoli cell lineage. (A) Comparison of the genomic region around *Sox9* in E15.5 Sertoli cells versus other cell types (fibroblast, ESC) and tissues (kidney, liver, heart, brain). The *Sox9-CFP* transgene is driven by the 3 kb TES enhancer (blue bar) located 13 kb upstream from the *Sox9* TSS. Enhancer activity was previously narrowed to a subregion of TES: the 1.3 kb TESCO element (Sekido and Lovell-Badge, 2008) (light-blue bar). DNaseI-seq identifies a DHS (black bar) spanning the TESCO element that is unique to the Sertoli cell lineage, consistent with the expression pattern of the transgene. Black bars above the track denote DHSs; Parzen scores are shown to the left of each track. (B) Based on their cell type specificity, E15.5 Sertoli cell DHSs were divided into unique, shared or common. Each group was subcategorized based on genomic location (promoter, gray; intergenic, purple; intronic, green; exonic, orange). (C) The percentage of DHSs overlapping CTCF sites based on the average overlap with eight previously generated CTCF ChIP-seq data sets (Shen et al., 2012).
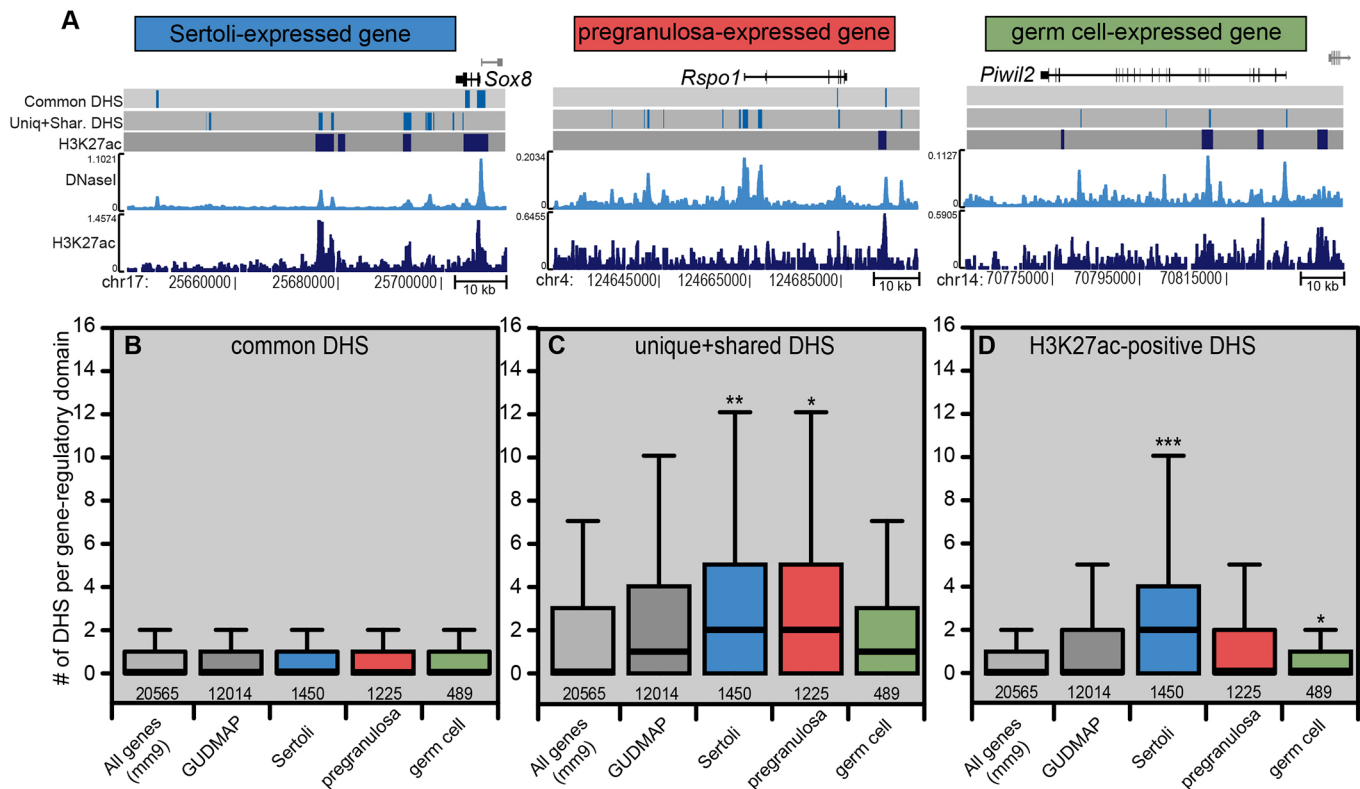
Fig. 3. Enrichment of DHSs near Sertoli and pregranulosa cell-expressed genes. (A) Using GREAT (McLean et al., 2010) each gene was assigned a gene-regulatory domain extending to the nearest 3′ and 5′ gene, but not overlapping the proximal promoter of either neighboring gene. Regulatory domains are shown for three examples: a Sertoli cell-expressed gene (*Sox8*), a pregranulosa cell-expressed gene (*Rpso1*) and a germ cell-expressed gene (*Piwil2*). Using data from E13.5 Sertoli cells, the three bars under the gene show the locations of common DHSs (top) and cell type-specific DHSs (middle) (vertical light-blue lines), or H3K27ac-positive DHSs (bottom; vertical dark-blue lines) for each region. The light-blue track shows the DNaseI-Seq Parzen score (autoscaled) and the dark-blue track shows H3K27 signal. Gene names are positioned adjacent to the TSS and other genes in the region are indicated in gray. (B-D) The correlation between E13.5 DHS sites and genes expressed in male or female supporting cells was plotted. The DHSs that mapped to each gene's regulatory domain were counted and the distribution of peaks per domain is shown as a box plot (outliers excluded). The results are shown for common DHSs (B), cell-type specific DHSs (C) and H3K27ac-positive DHSs (D). mm9 refers to all RefSeq genes (light gray); GUDMAP is the subset of mm9 genes that were analyzed in our previous microarray study [gray (Jameson et al., 2012b)]. Sertoli cell genes (blue), pregranulosa cell genes (red) and germ cells genes (green) categories are described in the supplementary Materials and Methods. Statistical significance was calculated using a two-sample Mann–Whitney (two-sided) test to compare each gene set to the GUDMAP reference set. *$P<1\times10^{-5}$, **$P<1\times10^{-10}$, ***$P<2.2\times10^{-16}$.

DHSs to one or two neighboring genes (Fig. 3A) and the number of DHSs (Fig. 3B,C) or H3K27ac-positive DHSs (Fig. 3D) was counted around genes enriched in these cell types.

As expected, common DHSs were not preferentially enriched around Sertoli-, pregranulosa- or germ cell-expressed genes (Fig. 3B). However, there was a significant increase in the number of cell type-specific (unique+shared) DHSs neighboring both Sertoli and pregranulosa cell-expressed genes (Fig. 3C). The similar enrichment of DHSs around Sertoli and pregranulosa cell-expressed genes may reflect the shared developmental origin of the supporting cell lineages, which have similar expression profiles just a few days prior to our analysis (E10.5) (Nef et al., 2005; Munger et al., 2013). DHSs around pregranulosa cell-expressed genes may define sites where repression complexes are bound to silence the alternative female pathway upon Sertoli cell differentiation.

H3K27ac-positive DHSs, which are indicative of active enhancers, were significantly enriched specifically in neighboring Sertoli cell-expressed genes (Fig. 3D), as expected. Additionally, we observed no enrichment of cell type-specific DHSs or H3K27ac-positive DHSs near germ cell-expressed genes, consistent with the unrelated origin of this cell lineage. Thus, using DNaseI-seq, we identified thousands of regulatory sites that are likely to be critical regulators of gene expression during Sertoli cell differentiation. Additionally, ChIP-seq

for H3K27ac classified these into active enhancers (associated with actively-expressed genes; H3K27ac-positive DHSs) or inactive enhancers (DHS-positive only) (Table S2).

Sertoli cell DHSs are enriched for TFs important for supporting cell development

To identify the TFs that bind putative Sertoli cell regulatory sites, we conducted computational analysis of the sequences in the Sertoli cell unique DHSs that did not overlap a promoter. We counted 6-mers in these DHSs, and used these as features in an L1 logistic regression classifier to classify these regions from length-matched sites flanking these DHSs (Koh et al., 2007) (Fig. 4A). A similar approach was shown to aid in the identification of sequence features underlying enhancer function in other mammalian systems (Lee et al., 2011). Our method is designed to identify enriched 6-mers found in a substantial fraction of DHSs. However, similar to other motif enrichment approaches, it is possible that it does not identify TFs that play a significant role in Sertoli cell biology, but bind to only a few locations.

Our analysis revealed 305 6-mers identified within Sertoli cell unique DHSs that are highly predictive of whether a region will be open specifically in Sertoli cells compared with flanking regions [area under receiver operating characteristic curve (AuROC)=0.82]
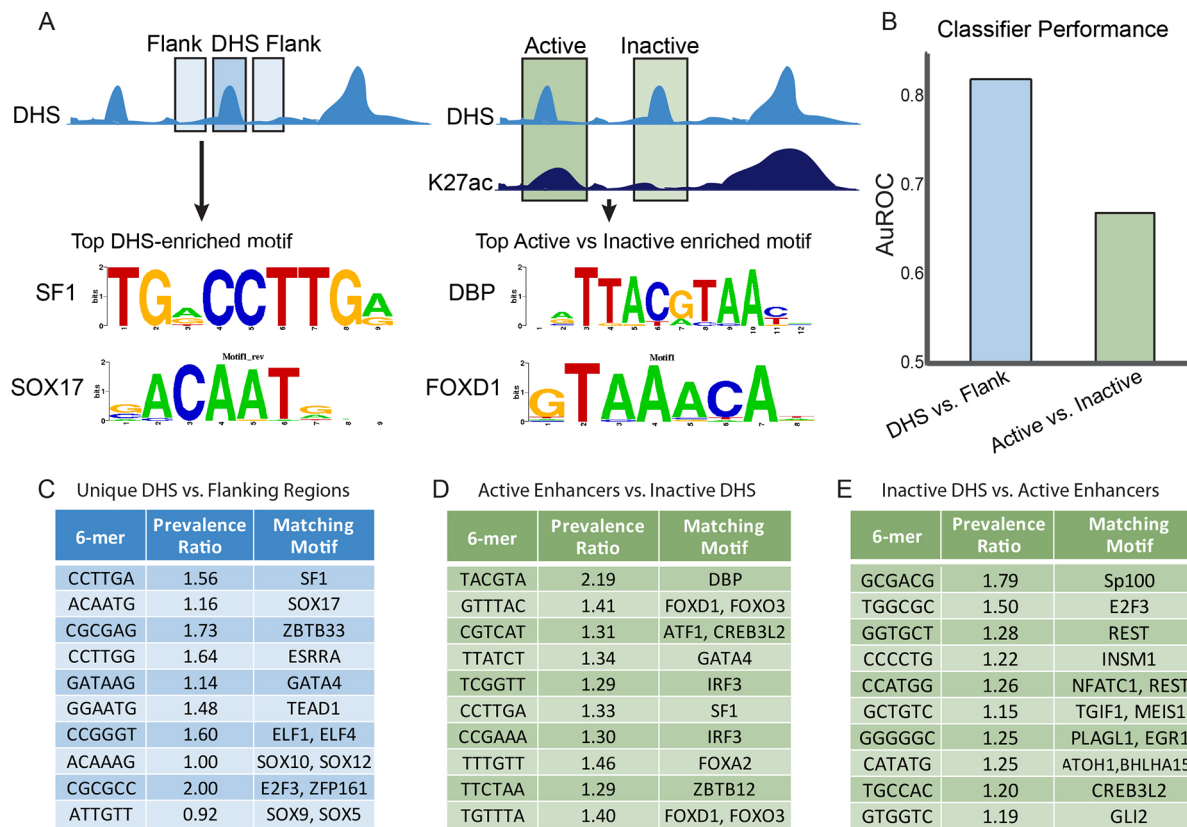
Fig. 4. TF-binding sites in active enhancers and inactive DHSs. (A) Schematic representation of the classification approach to identify motifs and TFs predictive of Sertoli cell DHSs. The top enriched motifs with known binding factors for sites predictive of DHS versus flank (left) and active versus inactive DHSs (right) are shown. (B) AuROC for different L1-logistic regression classifiers. Classifying active enhancers and inactive DHS against flanking regions shows high classifier performance (~0.82), while classifying active enhancers against inactive DHS shows lower performance (~0.67). (C-E) Top motifs driving predictive performance for the different classification tasks. TFs shown are best hits from the MEME suite's TOMTOM tool matching to the different 6-mers (Gupta et al., 2007). The prevalence ratio is defined as: the ratio of the average length normalized frequency of that 6-mer in DHSs unique to Sertoli cells relative to the flanking regions (C); the ratio of the average length normalized frequency of that 6-mer in active enhancers to inactive DHSs (D); the ratio of the average length normalized frequency of that 6-mer in inactive DHSs to active enhancers (E). The top 10 6-mers are shown in order of the absolute value of their regression coefficients. Only the first 6-mer matching to a particular TF is shown. TFs identified in D and E were identified in the same classifier, with those TFs shown in D more highly prevalent in active enhancers and those in E more prevalent in inactive DHSs. Table S3 contains the complete results for the analyses in C-E.

(Fig. 4B). To rule out the possibility of high classification accuracy due to repeats in flanking regions, we trained a classifier to distinguish Sertoli cell DHSs from a union set of unique DHSs from other cell types, including ES and fibroblast cells, and heart, kidney, liver and brain tissue. The classifier again showed high classification accuracy (AuROC=0.87). Furthermore, the 6-mers identified are similar between the two classification tasks, indicating that the features identified are robust predictors of Sertoli cell-specific DHSs (Table S3A,D).

We used TOMTOM (Bailey et al., 2009) to identify TFs that match these 6-mer motifs and found 75 6-mers matching known motifs. As multiple TFs have similar motifs, we used Sertoli cell gene expression (Jameson et al., 2012b) to identify the most likely candidates. The 6-mer most predictive of open chromatin in Sertoli cells matched the TF-binding motif for SF1 (Fig. 4C, Table S3), a factor required for early gonad formation in both sexes (Luo et al., 1994; Baba et al., 2014). Other prominent motifs identified include the SOX motif, which reflects the crucial role of SOX9 and SOX8 in the divergence of the Sertoli cell lineage from the undifferentiated progenitor cells (Chaboissier et al., 2004; Barrionuevo and Scherer, 2010), and the GATA motif, likely representing GATA4 (which is involved in early gonadal differentiation in both sexes) (Bouma et al., 2007b; Manuylov et al., 2008). Matches to SF1, SOX9 and

GATA4 motifs were also found to be statistically enriched in Sertoli cell-specific DHSs, supporting the validity of our method (Table S3E). Although less predictive of open chromatin, the FOX and TCF/LEF motifs were also identified (Table S3). FOXL2 (Schmidt et al., 2004; Ottolenghi et al., 2005) and Wnt signaling (which modulates gene expression through β-catenin binding with TCF/LEF factors) (Vainio et al., 1999; Chassot et al., 2008; Liu et al., 2009) are female-specific factors that are important for pregranulosa cell differentiation; enrichment of these motifs in Sertoli cell open chromatin may indicate sites requiring repression during Sertoli cell differentiation. Also identified was ELF1, an Ets-related TF. Ets family TFs can act downstream of FGF signaling (Sharrocks, 2001), which is crucial for repressing Wnt signaling to reinforce testis development (Kim et al., 2006; Jameson et al., 2012a). Additionally, we identified 6-mers matching TFs that are as yet not known to play a role in Sertoli cells, including TEAD1 and ZBTB33, as well as 6-mers that show no similarity to any known TF-binding motif (Table S3).

To determine whether distinct sequence features could differentiate active from inactive enhancers, we again used the L1-logistic regression classifier to classify the active enhancers (H3K27ac-positive DHS) from inactive DHSs (H3K27ac-negative DHS). The performance of the classifier was significantly reduced compared

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with our previous classifiers (AuROC=0.67), indicating that, despite the fact that active enhancers drive expression in Sertoli cells, sequence features between active enhancers and inactive DHSs are more similar than those between DHS and flanking regions (Fig. 4B). Inspection of the 6-mers that facilitate prediction of one class versus the other revealed 80 6-mers predictive of active enhancers and 64 6-mers predictive of inactive DHSs, many of which are not associated with a known TF. However, of the 6-mers predictive of an active enhancer, 25 did match a known motif. DBP, GATA4, FOXL1 and other FOX factor motifs were among the most predictive motifs for active enhancers (Fig. 4D). Although GATA4 is a well-known driver of Sertoli cell development (Bouma et al., 2007b), the role of DBP is unknown. Our identification of FOX motifs is consistent with a role of FOXL2 in repression of a *Sox9* enhancer (TESCO) in granulosa cells after birth (Uhlenhaut et al., 2009) and suggests that FOXL2 may play a similar role earlier in fetal development (see Discussion). Alternatively, several other FOX factors are expressed in both Sertoli and pregranulosa cells (Jameson et al., 2012b) that have yet to be characterized and could bind these sites.

In addition, the same analysis identified 20 6-mers with matching motifs that were more predictive of inactive DHSs compared with active enhancers. Although we did not identify motifs associated with known pregranulosa cell-specific TFs, the H3K27ac-negative DHSs likely represent a diversity of open chromatin sites, potentially associated with many genes that are repressed or poised in Sertoli cells, with or without sex-specific functions (Fig. 4E). Interestingly, some of the highest scoring 6-mers in this group were not associated with known binding factors.

The motif for another TF important for Sertoli cell development, DMRT1, did not come up as predictive of open chromatin in this analysis. However, we used available ChIP-seq data from E13.5 whole testes to analyze the enrichment of DMRT1-bound sites in

Sertoli cell open chromatin (Matson and Zarkower, 2012). We found that 70% of DMRT1 sites overlapped a DHS in our Sertoli cell dataset. However, the majority of DMRT1 sites overlapped promoters, with only 30% overlapping Sertoli cell unique and shared sites (Fig. S3). The exclusion of promoters from our 6-mer motif analysis may account for the absence of DMRT1 enrichment in DHSs analyzed.

DNaseI-Seq identifies a novel enhancer of Sertoli cell gene expression near *Wt1*

With the exception of the TESCO element, few distal regulatory elements have been identified for sex-determining genes. Our dataset provides an excellent resource with which to identify enhancers driving the expression of genes important in Sertoli cell differentiation. To demonstrate this, we performed a transient transgenic assay with a putative active enhancer of the *Wt1* gene (Fig. 5A,B). *Wt1* is initially expressed in multiple embryonic tissues, including the mesonephros and XX and XY gonadal somatic cells. Mutation of *Wt1*/*WT1* in mice and humans leads to kidney and genitourinary defects, including disorders of sexual development (Kreidberg et al., 1993; Eggers and Sinclair, 2012). After sex determination, *Wt1* expression becomes restricted to Sertoli cells in the testis and remains active in most somatic cells in XX gonads (Pelletier et al., 1991; Armstrong et al., 1993; Maatouk et al., 2012). We identified a DHS peak located 50 kb upstream of *Wt1* that was unique to E13.5 and E15.5 Sertoli cells compared with the other cell types we examined, and marked by H3K27ac, suggesting that it functions as an active enhancer. Finally, this region contained matches to several of the 6-mers identified in our motif analysis, including binding sites for SF1, SOX and FOX motifs.

Analysis of *in vivo* enhancer activity using a transgenic β-galactosidase reporter showed that the putative *Wt1* enhancer
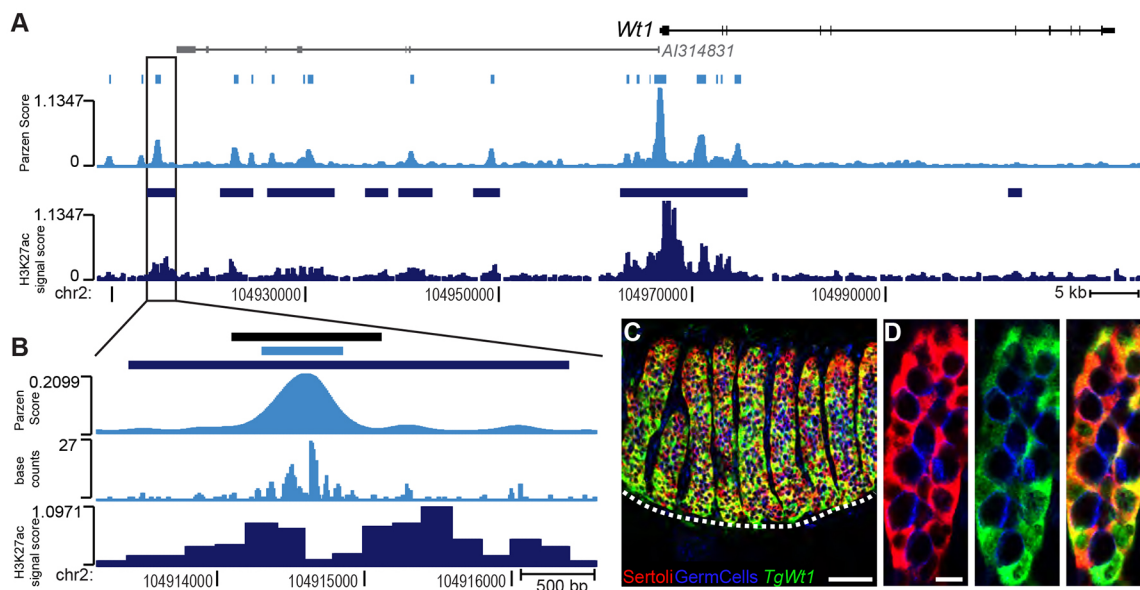

Fig. 5. Transient transgenic analysis of a DHS unique to Sertoli cells identifies a new Sertoli cell enhancer upstream of *Wt1*. (A) DNaseI-seq and H3K27ac ChIP-Seq data around the *Wt1* locus. Gene names indicate the TSS of the transcript. Nearby genes are indicated in gray. DNaseI-Seq data (Parzen score) and peak calls (boxes above track) are in light blue; H3K27ac ChIP-seq data and peak calls are in dark blue. A box surrounds the DHS chosen for *in vivo* analysis. (B) Detailed view of the DHS showing the DNaseI-seq Parzen score, the smoothed base counts (light blue) and the H3K27ac data (dark blue). The light-and dark-blue bars indicate the DNaseI-seq and H3K27ac peaks, respectively. The black bar marks the region cloned upstream of an *hsp68-LacZ* reporter cassette. (C) An E13.5 testis from a transgenic embryo showed β-galactosidase expression (*TgWt1*, green) specifically in Sertoli cells. Sertoli cells were labeled by AMH immunostaining (red) and germ cells by CDH1 (blue). The confocal image was taken using a 20× objective. Scale bar: 100 μm. (D) Confocal image of the sample in C taken with a 40× objective. Scale bar: 12.5 μm. All three panels show germ cells (blue); the left panel shows Sertoli cells (red); the middle panel shows β-galactosidase expression (green); the right panel shows the merge (yellow indicates co-expression of β-galactosidase and AMH).

was active in Sertoli cells of the testis (6/7 transient transgenic XY embryos; Fig. 5C,D and Fig. S5), where it exclusively localized in cells expressing the Sertoli cell marker AMH. Expression was also detected in somatic cells of the ovary (11/12 XX embryos; Fig. S5). However, no expression was detected in the mesonephros, embryonic kidney, heart or brain (Fig. 5C, Fig. S5 and data not shown) consistent with the cell-type specificity of this enhancer in our Sertoli cell datasets. These results demonstrate the power of our approach for the identification of distal enhancers that activate transcription in Sertoli cells.

DISCUSSION
Identification of Sertoli cell gene regulatory sites
Understanding the genetic networks that underlie developmental processes requires a comprehensive understanding of the genes involved as well as the regulatory mechanisms that modulate the expression of these genes. This is among a small number of studies to adapt epigenetic approaches, including DNaseI-seq and ChIP-seq, to address the resolution of cell fate in a cell type isolated from a complex developing tissue.

To discover cis-regulatory sequences during sex determination, we used DNaseI-seq to identify open chromatin sites and H3K27ac ChIP-seq to identify active enhancers in Sertoli cells. Using these data, we discovered TFs that might bind to these sites. We identified a novel active enhancer unique to Sertoli cells upstream of Wt1, and showed that it functions as a specific Sertoli cell enhancer in transgenic animals, as predicted. These data will enable future studies of cis-regulatory elements in Sertoli cells.

Although we were able to reduce the number of cells required for DNaseI-seq to 5 million cells, collection required more than 1 year, considering that a pair of E13.5 fetal testes contains only 10-20,000 Sertoli cells. Unfortunately, our attempts to perform DNaseI-seq on pregranulosa cells failed, possibly owing to the longer storage times required to collect sufficient numbers of cells (our pregranulosa cell reporter enabled purification of only 2-4000 cells per embryo). Rather than identifying sites of open chromatin based on DNaseI-seq, a new technique, ATAC-seq, uses a transposome-based method where the transposome preferentially integrates into sites of open chromatin (Buenrostro et al., 2013) and is highly correlative with DNaseI-seq data. Working on as few as 500 cells, this method presents an attractive alternative to enable studies of chromatin during cellular differentiation. Future studies will address whether a common chromatin architecture is maintained in differentiated XX and XY supporting cells, or whether these sites are eventually remodeled in a sexually dimorphic manner as differentiation proceeds.

DHSs adjacent to pregranulosa cell genes might indicate TF-mediated repression in Sertoli cell fate maintenance
In multipotent progenitor cell types, many developmentally regulated genes exist in an epigenetically poised state. Cis-regulatory elements of poised genes are marked by H3K4me1 and open chromatin (Heintzman et al., 2007). Upon differentiation, regulatory sites for active genes gain H3K27ac (Creyghton et al., 2010; Rada-Iglesias et al., 2011) and retain open chromatin, while repressed genes either remain poised or exhibit a loss of H3K4me1 and open chromatin (Whyte et al., 2012; Lara-Astiaso et al., 2014), perhaps rendering regulatory sites refractory to access by TFs.

In bipotential progenitor cells of the gonad, it is currently unknown whether genes that become sex-specific exist in an epigenetically poised state prior to sex determination. However, we hypothesized that, by E13.5, when sex-specific gene expression

patterns are well-established, open chromatin would be enriched near genes actively expressed in Sertoli cells. Although the enrichment of H3K27ac-positive active enhancers was associated with only Sertoli cell-expressed genes as expected, an intriguing finding was the widespread enrichment of DHSs around both Sertoli and pregranulosa cell-expressed genes in E13.5 differentiated Sertoli cells. There is evidence that many genes associated specifically with ovarian development are expressed in both sexes at the bipotential stage, suggestive of lineage priming (Jameson et al., 2012b), and initiation of the male pathway is characterized by extensive upregulation of the testis-pathway genes with simultaneous downregulation of ovarian-pathway genes. In contrast, transcriptional priming of pregranulosa progenitors towards the female pathway results in fewer transcriptional changes upon differentiation (Munger et al., 2009; Jameson et al., 2012b). One possible explanation for the retention of open chromatin near repressed pregranulosa cell genes in Sertoli cells is that loss of open chromatin sites occurs slowly, downstream of faster transcriptional changes. At a later developmental stage, fewer open chromatin sites might be associated with repressed gene loci. During ES cell differentiation, reduced H3K4me1 and open chromatin domains were observed near repressed genes within 48 h of differentiation (Whyte et al., 2012; Buecker et al., 2014). However, in multiple hematopoietic lineage descendants, the loss of open chromatin near repressed genes did not occur until late differentiation stages (Lara-Astiaso et al., 2014). We observed a similar enrichment of DHSs around both Sertoli and pregranulosa cell genes at E13.5 and E15.5 (Fig. S2), up to 4 days after peak Sry expression. Although it remains possible that a loss of open chromatin near repressed pregranulosa cell genes occurs later in adult Sertoli cells, an alternative possibility is that open chromatin regions persist near granulosa-expressed genes, perhaps reflecting the original bipotential progenitor capable of differentiating as a Sertoli or granulosa cell. Open chromatin near granulosa genes could be bound either by TFs that function to preserve a poised chromatin state or bound by repressive TFs that maintain the Sertoli cell fate. It is unclear whether this is a generalizable feature of cell populations that derive from a common bipotential progenitor.

An intriguing characteristic of gonadal supporting cells is that long after sex determination, some mutations cause transdifferentiation to the alternate cell fate. In the testis, Dmrt1 is required to prevent postnatal Sertoli to granulosa cell transdifferentiation by repression of several pregranulosa cell genes, including Foxl2, Esr2 and β-catenin (Matson et al., 2011; Minkina et al., 2014). In a reciprocal manner, mutation of Foxl2 causes granulosa-to-Sertoli cell transdifferentiation in the adult ovary (Schmidt et al., 2004; Ottolenghi et al., 2005). Our finding that Sertoli cells maintain open chromatin near many repressed granulosa genes might suggest that continued repression by TFs represents a mechanism for long-term maintenance of the Sertoli cell fate, and that these repressive mechanisms are established early during Sertoli cell development. Consistent with this, the TESCO enhancer of Sox9 is bound by FOXL2 in adult granulosa cells (Uhlenhaut et al., 2009), where Sox9 is silenced; however, it is bound by SRY/SOX9 and SF1 in Sertoli cells (Sekido and Lovell-Badge, 2008), where Sox9 is active. The use of the same cis-regulatory element to repress expression (via FOXL2 binding) in granulosa cells and activate expression (via SRY/SOX9 and SF1 binding) in Sertoli cells suggests that at least some cis-regulatory sites serve a dual purpose during sex-specific differentiation.

If the same regulatory sites act as enhancers in one cell type and repressors in the opposing cell type, then motifs for both male-promoting and female-promoting TFs would be located

within the active enhancers of Sertoli cell genes, available for competitive occupancy by male- or female-promoting TFs. Consistent with this, our motif analysis discriminating active versus inactive enhancers identified both the SOX and FOX motifs as predictive of active enhancers in Sertoli cells (Fig. 4 and Table S3). Furthermore, the lack of high classification accuracy from simple sequence features in active enhancers and inactive DHSs points to a complex and combinatorial regulatory code. Alternatively, differences in protein concentrations or post-translational modifications to regulators might explain the different transcriptional programs. The dual use of enhancers, such as TESCO, may underlie the ability of gonadal supporting cells to switch fates; however, it is currently unclear how extensive this mechanism is. Future studies mapping open chromatin sites in granulosa cells will reveal whether shared cis-regulatory sites represent a subset of gene regulatory mechanisms, or whether open chromatin sites are extensively shared between these functionally and morphologically distinct cell lineages.

Regulatory sites may harbor non-coding mutations that cause DSDs

The inability to provide a genetic diagnosis for the majority of individuals with a DSD has led to the development of more robust diagnostic tests to screen large regions of the genome for potential disease-causing mutations, including exome sequencing and copy number variation arrays (White et al., 2011; Arboleda et al., 2013; Baxter et al., 2015). This approach has revealed non-coding mutations upstream of SOX9 that result in DSDs (Kim et al., 2015). However, identifying non-coding mutations on a genome-wide scale remains difficult, and classification of non-coding mutations as potentially disease-causing is challenging, as these mutations do not directly alter the protein sequence. Recent studies have attempted to identify human enhancers for this purpose, using publicly available data from the NIH Roadmap Epigenomics Program (Roadmap Epigenomics Consortium et al., 2015). DNaseI-seq datasets for human fetal ovary and testis tissue have been used to identify putative enhancers (Ohnesorg et al., 2016). These tissues were presumably collected well after 4-6 weeks of gestation, the stage at which sex determination occurs in humans, and it is currently unclear whether enhancers that operate at the sex-determining stage are maintained in the late fetal stages. These issues can be addressed in mice; however, future studies will need to determine the extent of functional conservation between mouse and human enhancers. The genome-wide identification of regulatory sites that function during sex differentiation will enable more detailed studies on the regulation of sex-determining genes and will identify candidate regions that might harbor disease-causing mutations in humans with DSDs.

MATERIALS AND METHODS
Mouse strains, matings and cell/tissue isolation
To isolate Sertoli cells, *Sox9-ECFP* homozygous transgenic males (Kim et al., 2007b) were bred to CD-1 (Charles River) females in timed matings to generate E13.5 and E15.5 embryos. Following sorting by FACS, the cell pellet was snap-frozen and stored at −80°C for RNA-seq. For DNaseI-seq, cell pellets from the CFP-positive and CFP-negative fractions were resuspended in 250 µl of recovery-cell culture freezing media (Gibco) and slowly frozen to −80°C.

Mouse tissues (kidney, liver, heart, and brain) were collected from adult B6 mice. The B6 strain mouse fibroblast cell line was commercially available (Jackson Labs) and B6 strain ESCs were kindly provided by Ute Hochgeschwender (Duke University). For further details see the supplementary Materials and Methods.

Mice were housed in accordance with National Institutes of Health guidelines, and experimental protocols were approved by the Institutional Animal Care and Use Committee of Duke University Medical Center.

DNaseI-seq assay and data processing
DNaseI-seq assay was performed as previously described (Boyle et al., 2008a; Song and Crawford, 2010) with few modifications. DNaseI-seq libraries were prepared and data processed as previously described (Boyle et al., 2008b; Li and Durbin, 2009, Song and Crawford, 2010; Song et al., 2011). For further details see the supplementary Materials and Methods.

RNA preparation and RNA-seq data processing
E15.5 *Sox9CFP*-positive cells were pooled into three independent biological replicates containing ~1 million cells. RNA isolation was performed using the RNeasy Micro Kit according to the manufacturer's protocol (Qiagen). Poly-A enriched mRNA libraries were generated from 0.8-1 µg of RNA using the standard Illumina Tru-Seq V2 protocol and sequencing was carried out by the Duke Genome Sequencing and Analysis Core.

RNA-seq data was processed using Bowtie version 0.12.7 (Langmead et al., 2009) and RSEM version 1.2.0 (Li and Dewey, 2011) as described in the supplementary Materials and Methods. RSEM expected counts were quartile normalized and square-root transformed.

TSS and regulatory domain assignments
Transcriptional start and end sites were downloaded from the UCSC Genome browser and used to generate regulatory domains using GREAT (McLean et al., 2010). For further details see the supplementary Materials and Methods.

Genomic location assignments
DHSs were assigned to specific genomic locations using previously published methods (Song et al., 2011). For further details see the supplementary Materials and Methods.

Cell type specificity categorization
Cell type specificity was determined by comparing DHSs from DNaseI-seq datasets from other cell types using the BedTools Software suite (Quinlan et al., 2010). For further details see the supplementary Materials and Methods.

CTCF overlap
We used previously published CTCF ChIP-seq datasets (Shen et al., 2012) and compared these against Sertoli-unique, Sertoli-specific and Sertoli-common DHSs using BedTools. For further details see the supplementary Materials and Methods.

DMRT1-binding site enrichment analysis
We used previously published DMRT1 ChIP-seq data (Krentz et al., 2013) and assessed overlap with Sertoli DHSs using BedTools. For further details see the supplementary Materials and Methods.

Enrichment of DHSs in Sertoli cell-expressed genes
To calculate enrichment, we assessed the number of DHSs that were assigned to each gene's regulatory domain. DHSs were divided as common, unique+shared or active enhancers (H3K27ac-positive). Significance was calculated using a two-sided Mann-Whitney test. For further details see the supplementary Materials and Methods.

Transient transgenics, immunocytochemistry and imaging
A putative regulatory region upstream of *Wt1* was amplified and cloned into the *Hsp68-LacZ* reporter vector (Addgene; Plasmid #33351). DNA was prepared for zygote injection, and resuspended in EmbryoMax Injection Buffer (Millipore, MR-095-10F). Pronuclear injections into B6SJLF1/J zygotes were performed by the Duke Transgenic Core Facility to generate transient transgenics. Embryos were dissected at E13.5 and genotyped for the *LacZ* gene For immunostaining, fixed gonads were processed as previously described

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(Maatouk et al., 2012). Antibodies were used at the following dilutions: rat-anti-CDH1, 1:250 (Zymed, 13-1900); rabbit-anti-β-galactosidase, 1:10,000 (MP Biomedicals, 55976); goat-anti-MIS/AMH, 1:250 (Santa Cruz, sc-6886); Alexa Fluor 488-anti-rat, 1:500 (Molecular Probes, A21208); Cy3-anti-rabbit, 1:500 (Jackson ImmunoResearch Laboratories, 711-165-15); Alexa Fluor 647-anti-goat, 1:500 (Molecular Probes, A21447). For further details see the supplementary Materials and Methods.

ChIP-seq assay and data processing

For ChIP-seq, FACS-purified Sertoli cells were pelleted, resuspended, crosslinked and stored at −80°C. Briefly, 1 million cells were pooled from multiple sorts, washed, lysed, sonicated and incubated with bead-antibody complexes with 2.5 µg antibody (Rabbit-anti-H3K27ac; Abcam ab4729). Library preparation was performed on immunoprecipitated chromatin using the Rubicon ThruPLEX FD kit according to the manufacturer's protocol. Sequencing was performed at Duke's Genome Sequencing and Analysis core facility.

ChIP-seq reads were uniquely aligned with Bowtie (Langmead et al., 2009); peaks were called with SICER (Zang et al., 2009) using the input track as the control. For further details see the supplementary Materials and Methods.

Sequence analysis to identify predictive 6-mers and matching motifs

Enriched motifs were identified by incorporating previous methods (Lee et al., 2011; Natarajan et al., 2012). 6-mers and their reverse complements, were counted in each region, normalized by the length of the region, and used as features for an L1-norm sparse logistic regression classifier (Koh et al., 2007). Ten randomized iterations of fourfold cross-validation were performed to generate 40 different partitions of the data. 6-mers that showed non-zero regression coefficients in over 75% of the partitions were deemed to be significant and are shown in Table S3A-D. 6-mers were matched to known TF PWMs with TOMTOM (Gupta et al., 2007) using the JASPAR core vertebrate motifs, the UNIPROBE database and those generated by Jolma et al. (Bryne et al., 2007; Jolma et al., 2013; Hume et al., 2015). The SF1 (Baba et al., 2014) and DMRT1 (Krentz et al., 2013) motifs were included. For further details see the supplementary Materials and Methods.

Analysis of significance of motif matches

We used FIMO from the MEME Suite (Grant et al., 2011) to scan different regions of the genome. Length normalized motif match scores were used in a one-sided Mann-Whitney U-test to test for significance. For further details see the supplementary Materials and Methods.

Data availability

Datasets (including DNaseI-seq, ChIP-seq and RNA-seq) are publicly available in GEO (Accession Number GSE53076).

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.142554.supplemental

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Supplemental Materials and Methods

Mouse Strains, Matings, and Cell/Tissue Isolation

Transgenic *Sox9-ECFP* mice (Kim et al., 2007b) were maintained on a C57BL/6J (B6) genetic background. To isolate pre-Sertoli cells, *Sox9-ECFP* homozygous transgenic males were bred to CD-1 (Charles River) females in timed matings to generate E13.5 and E15.5 embryos. Noon of the day a vaginal plug was observed was defined as E0.5. *Sox9-ECFP*$^{+/-}$ embryos were dissected and testes removed from the adjacent mesonephros. Testes from one or more litters were pooled together, incubated in 500 µl of 0.25% Trypsin-EDTA (Gibco) plus 0.25% Collagenase at 37°C for 8-10 minutes. The Trypsin-EDTA was removed and the tissue rinsed with 1X PBS with 3% BSA, and then dissociated by gentle pipetting in 500 µl 1X PBS with 3% BSA. Dissociated cells were passed through a cell strainer (BD Falcon) to ensure a single cell suspension.

FACS was performed by the Duke Comprehensive Cancer Center Flow Cytometry Shared Resource facility on a BD FACStar sorter, running at 12 psi, using a water-cooled Coherent argon laser tuned to 458 nm run at 50 mW. CFP emission was collected with a 485/22 bandpass filter. Following sorting, the cells were spun down at 2 krpm for 20 min at 4°C and the supernatant removed. For RNA-seq, the cell pellet was snap-frozen and stored at -80°C. For DNaseI-seq, cell pellets from the CFP-positive and CFP-negative fractions were resuspended in 250 µl of Recovery-Cell Culture Freezing Media (Gibco) and slowly frozen to -80°C.

Mouse tissues (kidney, liver, heart, and brain) were collected from adult B6 mice, flash frozen and then pulverized before use. The mouse fibroblast cell line was derived from adult B6 mice (Jackson Labs). ESCs, also of the B6 strain, were kindly provided by Ute Hochgeschwender (Duke University) and were grown on gelatinized plates in the absence of a feeder layer or matrigel. To harvest ESCs, plates were washed with 1X PBS and treated with 0.25% Trypsin-EDTA for 7-10 minutes at 37°C. An equal

volume of medium (containing 10% FBS) was then added to the plates to stop trypsinization. Cells were collected and pipetted up/down to get a single cell suspension and were centrifuged at ~1.2 krpm for 10 minutes. All medium was removed from the cell pellet.

DNaseI-seq Assay and Data Processing

Because our experiments were severely limited by the ability to collect large numbers of FACS purified pre-Sertoli cells, DNaseI digestion optimization was first carried out on differing amounts of gonadal cells collected by FACS (CFP-negative fractions). Attempts using 1-5 million cells were performed and limited success was achieved with 3 million cells; however, consistent digestion patterns were observed when using 5 million cells. Once 5 million CFP-positive cells were collected, the DNaseI-seq assay was performed as previously described (Boyle et al., 2008a; Song et al., 2010) with few modifications. Incubation of FACS purified pre-Sertoli cells with low concentrations of exogenous DNaseI enzyme was found to severely degrade the DNA; therefore DNase digestion was performed using endogenous nucleases. Briefly, cells were lysed and incubated at 37°C for various times (5 min to 1 hour) and optimal DNase digestion was confirmed by pulse field gel electrophoresis (Song et al., 2010). DNaseI-seq libraries were then prepared as previously described (Boyle et al., 2008a; Song et al., 2010) and sequenced (three lanes) on the Illumina GAII platform by the Duke Genome Sequencing and Analysis Core. DNaseI-seq data was processed as previously described (Song et al., 2011). Briefly, sequences were aligned to the mouse reference genome (UCSC mm9) using BWA (Li et al., 2009), reads were filtered to remove PCR amplification artifacts (associated with library processing), base-pair signal (Parzen score) was generated using F-seq (Boyle et al., 2008b) and discrete peaks corresponding to DHSs were called.

RNA preparation and RNA-seq Data Processing

E15.5 *Sox9CFP*-positive cells were collected from ~165 embryos and pooled into 3 independent biological replicates, each containing ~1 million cells. RNA isolation was performed using the RNeasy Micro Kit as previously described (Jameson et al., 2012b) and according to the manufacturer's protocol (Qiagen). The RNA was DNaseI digested and eluted from the column with 14 µl of RNase-free water. QC and quantitation was performed on the Thermoscientific NanoDrop 2000 and the Agilent Bioanalyzer (NanoDrop results: ~135-160 ng/µl; Bioanalyzer results: ~90-110 ng/µl).

Library preparation and sequencing was carried out by the Duke Genome Sequencing and Analysis Core. Poly-A enriched mRNA libraries were generated from 0.8-1 µg of RNA using the standard Illumina Tru-Seq V2 protocol, then quantitated on the Agilent Bioanalyzer and adapted with index 23, 25 or 27. 7pM of the resulting library pool was run in a single Illumina HiSeq2000 lane to generate 50 bp single-end reads. Base calls were performed using CASAVA (version 1.8.2), which provides only pass-filtered reads. Output files for each biological replicate were concatenated. For each sample, 57-63 million reads were generated and over 96% of reads had a quality score equal to or greater than Q30, with a mean quality score of 38, therefore no further filtering was performed.

RNA-seq data was processed using Bowtie version 0.12.7 (Langmead et al., 2009) and RSEM version 1.2.0 (Li et al., 2011). The rsem-prepare-reference command was used to generate Bowtie-compatible index files for the UCSC mm9 transcriptome using UCSC gene transcript annotations and genome fasta files (random chromosomes were removed). Reads were aligned to the prepared reference transcript file using Bowtie with the following options: -v 3 -a -m 100 --best –strata. This resulted in ~83% of the reads having at least one reported alignment to the transcriptome.

Gene- and isoform-level abundance was estimated using the rsem-calculate-expression command. The TruSeq library fragment size distribution was estimated based on the Agilent Bioanalyzer

report; the fragment-length-mean and fragment-length-sd were set to 350 and 100, respectively. Expected counts were then quartile normalized and square-root transformed prior to further analysis.

TSS and regulatory domain assignments

To determine the overlap of DHSs with the TSS of each gene in the genome, a file was generated that assigned a single transcriptional start site to each gene. The transcriptional start and end for each gene was extracted from the refgene table downloaded from the UCSC genome browser (mm9). For genes that had multiple isoforms, the transcript's 5'-most (TxStart) and/or 3'-most (TxEnd) ends were used, resulting in one TxStart/TxEnd for each gene in the refgene table.

Gene-regulatory domains were generated by using GREAT (McLean et al., 2010) to assign each DHS to the one or two nearest genes (excluding non-coding RNAs). Briefly, for each gene a domain was extended up to 2 Mb from the TxStart and TxEnd to the next nearest gene in the 5' and 3' directions, but stopping at -5 kb from neighboring gene's TSS, leaving the proximal promoter assigned only to the nearest gene.

Genomic location assignments

Genomic locations were assigned to DHSs using previously published methods (Song et al., 2011). Briefly, DHSs were assigned to the first of the following categories that it overlapped: (1) promoter: overlaps a TSS or 2 kb upstream; (2) 5' exon/intron: overlaps the first exon/intron; (3) intragenic exon/intron: overlaps an internal exon or intron; (4) 3' exon: overlaps the last exon; or (5) intergenic: not overlapping any previous category. 5' exon, intragenic exon and 3' exon were then combined into a single category: exonic. 5' intron and intragenic intron were combined into a single category: intronic.

Cell type specificity categorization

Cell type specificity was determined by comparing DHSs from seven DNaseI-seq datasets: Sertoli, fibroblast, ESC, kidney, liver, heart and brain. DHSs were categorized into three groups: Sertoli-unique (only found in the Sertoli DNaseI-seq data), Sertoli-specific (present in Sertoli and up to 5 other cell types) and Sertoli-common (present in all seven cell types). Sequential intersections were performed for each of the DNaseI-seq datasets using the intersectBed command from the BedTools Software suite (version 2.17.0) (Quinlan et al., 2010). In each case (unique, specific or common), the boundaries for the Sertoli DHSs were maintained.

CTCF overlap

We used CTCF ChIP-seq data that was available on the Mouse Encode Project at Ren Lab website (http://chromosome.sdsc.edu/mouse/download.html) (Shen et al., 2012). CTCF ChIP-seq data from four adult tissues (liver, lung, spleen and testis) and four E14.5 tissues (brain, heart, liver and limb) was downloaded, and CTCF binding sites were extended 100 bp (50 bp in the 5' and 3' direction). Pairwise intersections were performed for each of the eight CTCF datasets with Sertoli-unique, Sertoli-specific and Sertoli-common DHSs using intersectBed (Quinlan et al., 2010) and at least 25 bp of overlap was required to be considered overlapping. The percentage of DHSs that overlapped a CTCF binding site was reported as the average and standard deviation of overlap across all 8 cell types (see Figure 2).

DMRT1 binding site enrichment analysis

To look for enrichment of DMRT1 binding sites in Sertoli DHSs, the overlap of DMRT1 ChIP-seq binding sites from E13.5 testes (Krentz et al., 2013) with Sertoli DHSs was analyzed using intersectBed (Quinlan et al., 2010). 25 bp of overlap was required to be considered overlapping. Sertoli DHSs were subdivided into "unique", "shared", or "common" based on their cell-type specificity as described above.

Enrichment of DHSs at Sertoli-expressed genes

To determine whether Sertoli DHSs were enriched near Sertoli-expressed genes, the number of DHSs that mapped to each gene's regulatory domain were counted (using intersectBed; (Quinlan et al., 2010) for the following categories of Sertoli DHS: common, unique+shared and active enhancers (H3K27ac-positive DHSs). Overlap was analyzed with the following categories of genes: mm9 (refers to all genes, isoforms removed as described above); GUDMAP (the subset of mm9 genes that were analyzed in our previous microarray study; (Jameson et al., 2012b); Sertoli (genes expressed >1.5 fold higher in E13.5 Sertoli cells compared to E13.5 pregranulosa cells), pregranulosa (genes expressed >1.5 fold higher in E13.5 pregranulosa cells compared to E13.5 Sertoli cells), and germ cells (genes expressed >1.5 fold higher in E13.5 male or female germ cells compared to all other gonadal cell lineages). Statistical significance was calculated using a two-sample Mann-Whitney (two-sided) test to compare the distributions for each gene set to the GUDMAP reference set.

Transient transgenics, immunocytochemistry and imaging

A putative regulatory region (UCSC mm9 coordinates chr2:104914099-104915125) upstream of *Wt1* was amplified by PCR and cloned into the NotI site of the *Hsp68 –LacZ* reporter vector (obtained from Addgene; Plasmid #33351). Cloning was carried out using In-Fusion HD (Clonetech). To prepare DNA for zygote injection, 50 μg of the *TgWt1* plasmid was linearized with NotI-HF and HindIII and gel purified by electroelution. The DNA was phenol-chloroform extracted, ethanol precipitated and resuspended in EmbryoMax Injection Buffer (Millipore, MR-095-10F). The DNA was further purified on a DNA-cleanup column (Qiagen) and eluted again in EmbryoMax Injection Buffer. Pronuclear injections into B6SJLF1/J zygotes were performed by the Duke Transgenic Core Facility to generate transient transgenics.

Embryos were dissected at E13.5 and the embryonic tail was removed for PCR genotyping to detect the *LacZ* gene (Primers (5'-3'): F-ATCCTCTGCATGGTCAGGTC and R-CGTGGCCTGATTCATTCC).

Gonads were carefully dissected from embryos and fixed in 4% paraformaldehyde for several hours or overnight at 4°C. The remaining embryo bodies were fixed in 4% paraformaldehyde for 8 minutes, washed in X-gal wash buffer (2mM MgCl$_2$, 0.2% NP-40 in 1X PBS) and then incubated overnight at 37°C in X-gal staining solution (5mM potassium ferrocyanide, 5mM potassium ferricyanide, 1mg/ml X-gal in X-gal wash buffer).

For immunostaining, fixed gonads were washed three times in 1X PBS and incubated in blocking solution (10% FBS, 3% BSA and 0.1% Triton-X-100 in 1X PBS) for 1 hr at room temperature. Blocking solution was replaced with primary antibodies diluted in blocking solution and incubated overnight at 4°C. The next morning, samples were washed three times in washing solution (1% FBS, 3% BSA and 0.1% Triton-X-100 in 1X PBS) followed by one-hour incubation with blocking solution. Samples were then incubated with secondary antibodies, diluted in blocking solution, overnight at 4°C. Following three washes, samples were mounted in DABCO (2.5% 1,4, diazagicyclo octane, 90% glycerol in 1X PBS). Images were captured on a Leica SP2 confocal microscope.

Primary and secondary antibodies were used at the following dilutions: rat-anti-CDH1, 1:250 (Zymed, 13-1900); rabbit-anti-β-galactosidase, 1:10,000 (MP Biomedicals, 55976); goat-anti-MIS/AMH, 1:250 (Santa Cruz, sc-6886); Alexa Fluor 488-anti-rat, 1:500 (Molecular Probes, A21208); Cy3-anti-rabbit, 1:500 (Jackson ImmunoResearch Laboratories, 711-165-15); Alexa Fluor 647-anti-goat, 1:500 (Molecular Probes, A21447).

ChIP-seq assay and data processing

For ChIP-seq analysis, FACS purified Sertoli cells were pelleted, resuspended in 360 µl of PBS and cross-linked with 10 µl of 37% formaldehyde at room temperature for 10 minutes. Cross-linking was stopped by addition of 46.3 µl of 1M glycine for 5 minutes at room temperature. Cells were then pelleted, supernatant was removed and stored at -80°C. 1 million cells were pooled from multiple sorts

washed twice in 500 µl of PBS with protease inhibitors. Cells were resuspended in 500 µl of lysis buffer (50mM Tris-HCL, 10mM EDTA, 1% SDS and protease inhibitors) and sonicated with a Branson 450 Sonicator (output power of 3, duty cycle of 30% for 16 cycles of 30 seconds with 1 minute rest time between sonications). Bead-antibody complexes were prepared by incubating 30 µl of dynabeads (Protein A; Life Technologies 10002D) with 2.5 µg antibody (Rabbit-anti-H3K27ac; Abcam ab4729).

The sonicated lysate was spun down at 4°C for 10 minutes at 10 krpm with 40 µl of the supernatant set aside as input and 200 µl transferred to tubes containing pre-incubated bead-antibody complexes. We added 700 µl of ChIP Dilution Buffer (CDB) (1 % Triton X-100 (Sigma T8787), 2mM EDTA, 150mM NaCl, 20mM Tris (pH=8.0)) with protease inhibitors to IP tubes, and incubated overnight at 4°C. 160 µl of CDB and 8 µl of 5M NaCl were added to the input tube which was incubated at 65°C overnight. The following day, IP tubes were washed as follows: Once with Wash Buffer 1 (50mM Tris HCl, 1mM EDTA, 150 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 0.1% Sodium deoxycholate), twice with Wash Buffer 2 (50mM Tris HCl, 1mM EDTA, 500 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 0.1% Sodium deoxycholate), once with Wash Buffer 3 (10mM Tris HCl, 1mM EDTA, 1% NP-40, 1% Sodium deoxycholate, 250mM LiCl), twice with Wash Buffer 4 (50mM Tris HCl, 1mM EDTA, 500 mM LiCl, 1% NP-40, 0.7% Sodium deoxycholate), twice with TE buffer (pH=8.0). All washes were done in 1 ml, with added protease inhibitors, at 4°C for 5 minutes. Solutions for wash buffers were modified from the protocols posted on the Epigenomics Roadmap website.

DNA-protein complexes were eluted twice from the beads with 100 µl elution buffer (100mM sodium bicarbonate, 1% SDS, 8mM NaOH). 8 µl of 5M NaCl was added to the eluates, as well as the input tube, and incubated at 65°C overnight. Samples were treated with 1 µl RNase-cocktail (Life Technologies AM2286) for 30 minutes at 37°C, then 4 µl of 0.5M EDTA, 8 µl of 1M Tris and 1 µl of 10 mg/ml Proteinase K was added for 60 minutes at 45°C. Finally, DNA was purified using PCR purification columns (Qiagen; 28104).

For library preparation for sequencing, DNA was concentrated using a vacuum centrifuge to ~10 µl. 10 µl of IP DNA and 1 µl of input DNA was used in the library preparation using the Rubicon ThruPLEX FD kit according to the manufacturer's protocol. Size selection of smaller size amplified DNA was done with SPRI beads (Agencourt AMPure XP A63880) at 0.6x concentration. Sequencing was performed at Duke's Genome Sequencing and Analysis core facility on the Illumina HiSeq2000/2500

ChIP-seq reads were aligned with Bowtie (Langmead et al., 2009) with only uniquely aligning reads used for future processing. Peaks were called with SICER (Zang et al., 2009) with enrichment called for the histone modifications using the input track as the control. The species variable was set to mm9, redundancy threshold to 2, window size to 200, fragment size to 150, effective genome fraction to 0.7, gap size to 600 and FDR to 0.01. Peaks found in both replicates were used to identify active enhancers and inactive DHSs.

Sequence analysis to identify predictive 6-mers and matching motifs

To identify enriched motifs, a discriminative classification approach using sequence features was performed incorporating previous methods (Lee et al., 2011; Natarajan et al., 2012). 6-mers, with reverse complements counted as the same 6-mer, were counted in each region, normalized by the length of the region, and used as features for an L1-norm sparse logistic regression classifier (Koh et al., 2007). Ten randomized iterations of 4-fold cross-validation were performed to generate 40 different partitions of the data. 6-mers that showed non-zero regression coefficients in over 75% of the partitions were deemed to be significant and shown in Table S1A-D. We evaluated the 6-mers identified as consistently important in our classifiers by two metrics. First, we used the regression coefficient averaged over the cross-validation iterations. The regression coefficient in a logistic regression classifier is the log odds ratio as a result of a unit increase in the variable. In addition, we calculated the prevalence ratio of the 6-mers. Prevalence ratio is defined as the ratio of the average length normalized 6-mer frequency between Sertoli specific DHS (active enhancer) vs. flanking regions (inactive DHS). 6-

mers were matched to known TF binding sites with TOMTOM (Gupta et al., 2007) using the following options: -no- -min-overlap 5 -mi 1 -dist pearson -evalue -thresh 0.5 -query-pseudo 0.01. The motif database included motifs from the JASPAR core vertebrate motifs, the UNIPROBE database and those generated by Jolma et al. (Bryne et al., 2008; Jolma et al., 2013; Hume et al., 2015). Additionally, the SF1 (Baba et al., 2014) and DMRT1 (Krentz et al., 2013) motifs were included.

Analysis of significance of motif matches

To assess significance of motif matches in different regions, we used FIMO from the MEME suite (Bioinformatics 27:1017) with default settings to scan different regions of the genome with three motifs SOX9, SF1, and GATA4. The score of the motif matches were then summed up across each region and length normalized. We used a one-sided Mann-Whitney U test to test for significance between the length normalized motif matches in each region.
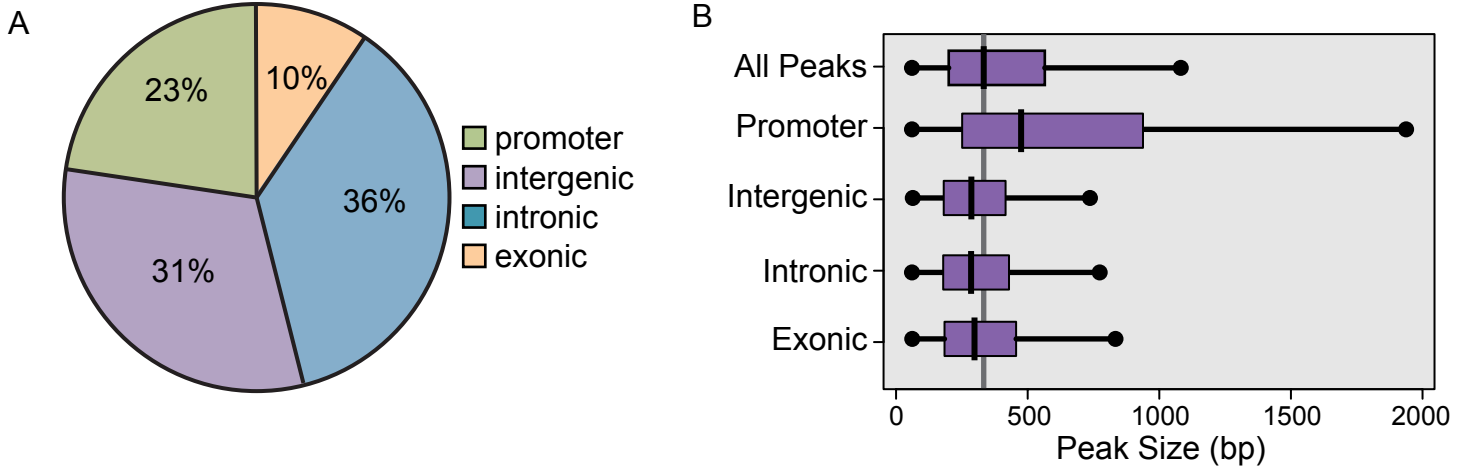
Supplemental Figures

A

B

Figure S1. Location and size distribution of E15.5 DHSs. (A) Genomic location distribution analysis of all DHSs. Peaks were categorized by overlap with specific genomic features (promoter, exonic, intronic or intergenic) as described in the Online Methods. (B) A boxplot of the peak size distributions. Purple boxes indicate the middle 50% of peaks, lines mark the lower (left) and upper (right) 25%. Outliers are not shown. The vertical gray line indicates the median length for all DHS peaks (330bp).
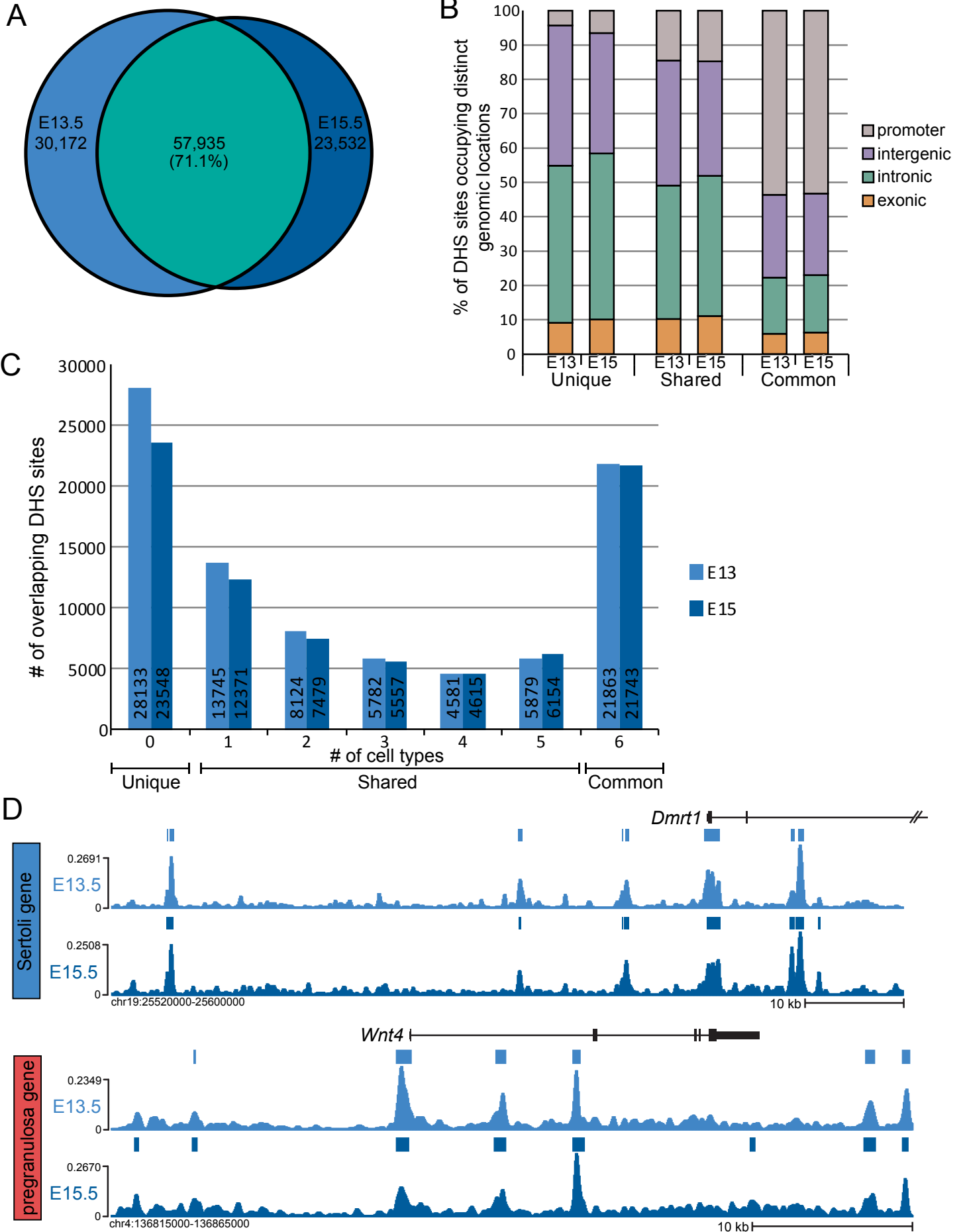
Figure S2. Concordance between E13.5 and E15.5 DNaseI-seq datasets. (A) Using bedtools (Quinlan et al., 2010), intersection of the E13.5 and E15.5 DHSs shows >65% overlap between the 2 datasets. (B) Unique, Shared and Common DHSs have a similar genomic distribution for both the E13.5 and E15.5 datasets. (C) Distribution of the number of DHSs that are found in other somatic cells types (primary skin fibroblasts, embryonic stem cells, adult kidney, liver, heart and brain). The x-axis shows the number of additional cell types where the DHS is present (i.e. '1' indicates DHSs present in E13.5 or E15.5 Sertoli cells and one other somatic cell type). Numbers within the bars indicate the number of DHSs with that category. DHSs that were only present in Sertoli cells ("0") were classified as Unique; DHSs present in Sertoli cells and 1-5 other cells types were classified as Shared; DHSs present in all 7 cells types were classified as Common. (D-E) Sample genomic loci comparing DNaseI-seq data between E13.5 and E15.5. Similar DHS distributions were observed at both developmental stages including at Sertoli-expressed genes such as *Dmrt1* (D), and pregranulosa-expressed genes such as *Wnt4* (E).
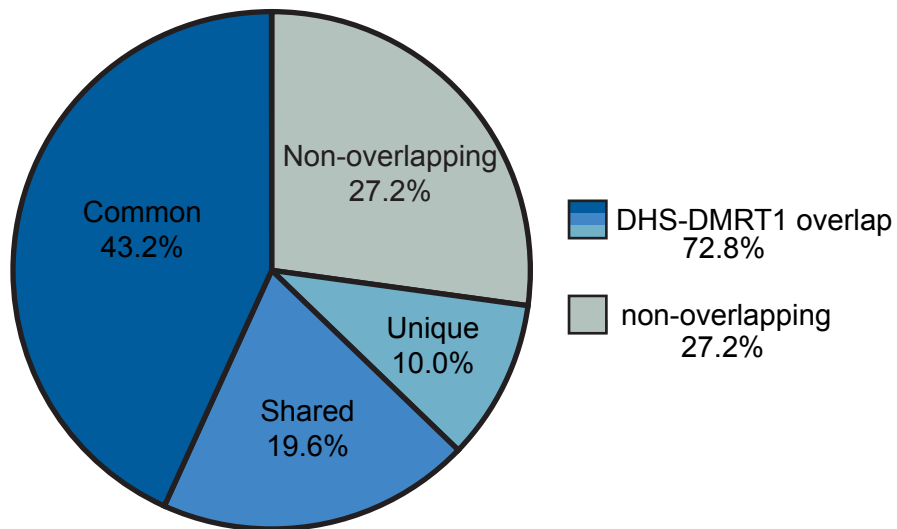
Figure S3. Enrichment of DMRT1 sites in Sertoli DHSs. The percentage of E15.5 DMRT1 binding sites

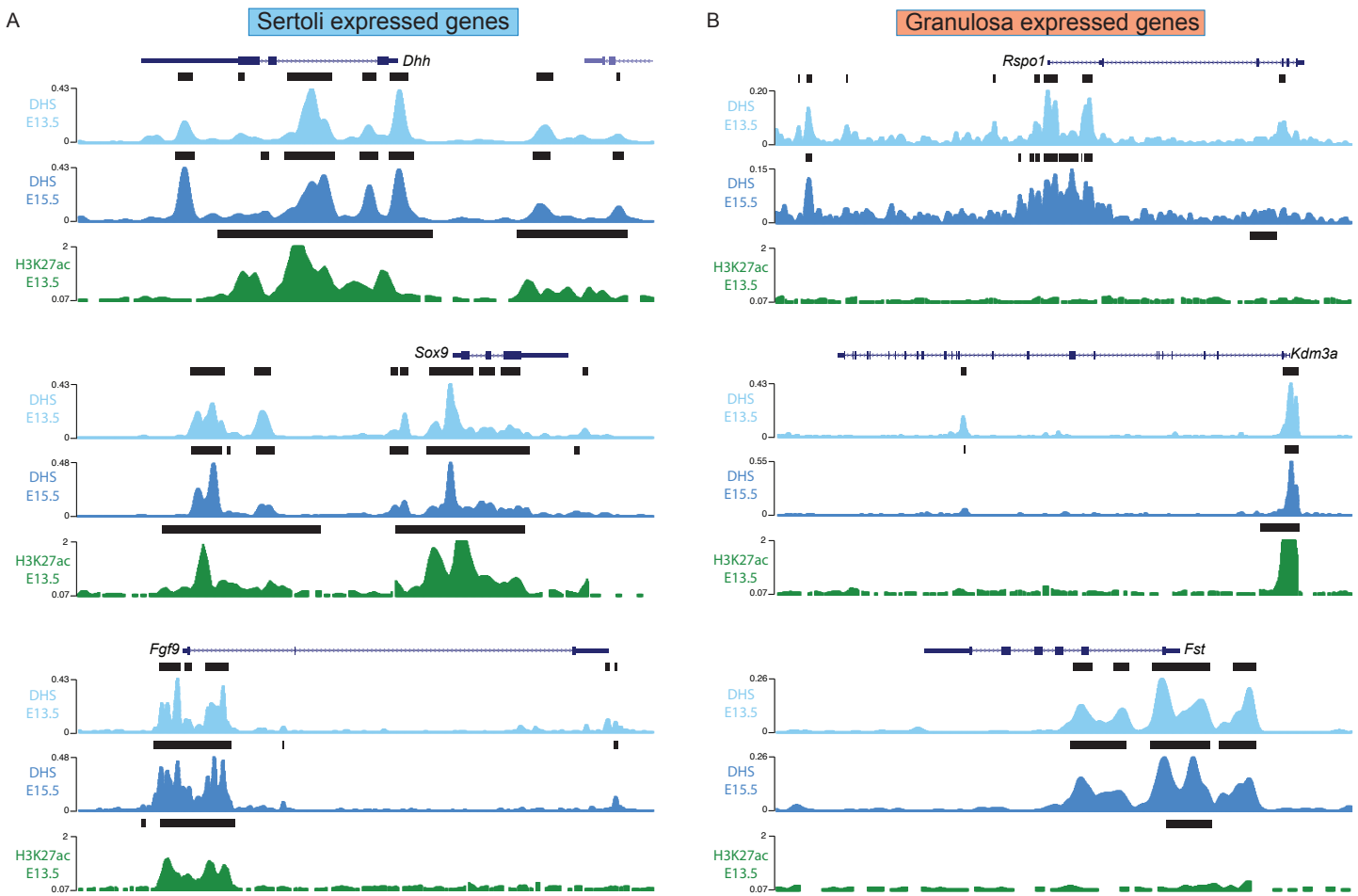(Krentz et al., 2013) that overlap Sertoli unique, shared; or common DHSs.

Figure S4. DHS and H3K27ac sites around select genes. DHS and H3K27ac sites around select (A) Sertoli

and (B) granulosa expressed genes. Genes were identified from expression data published previously
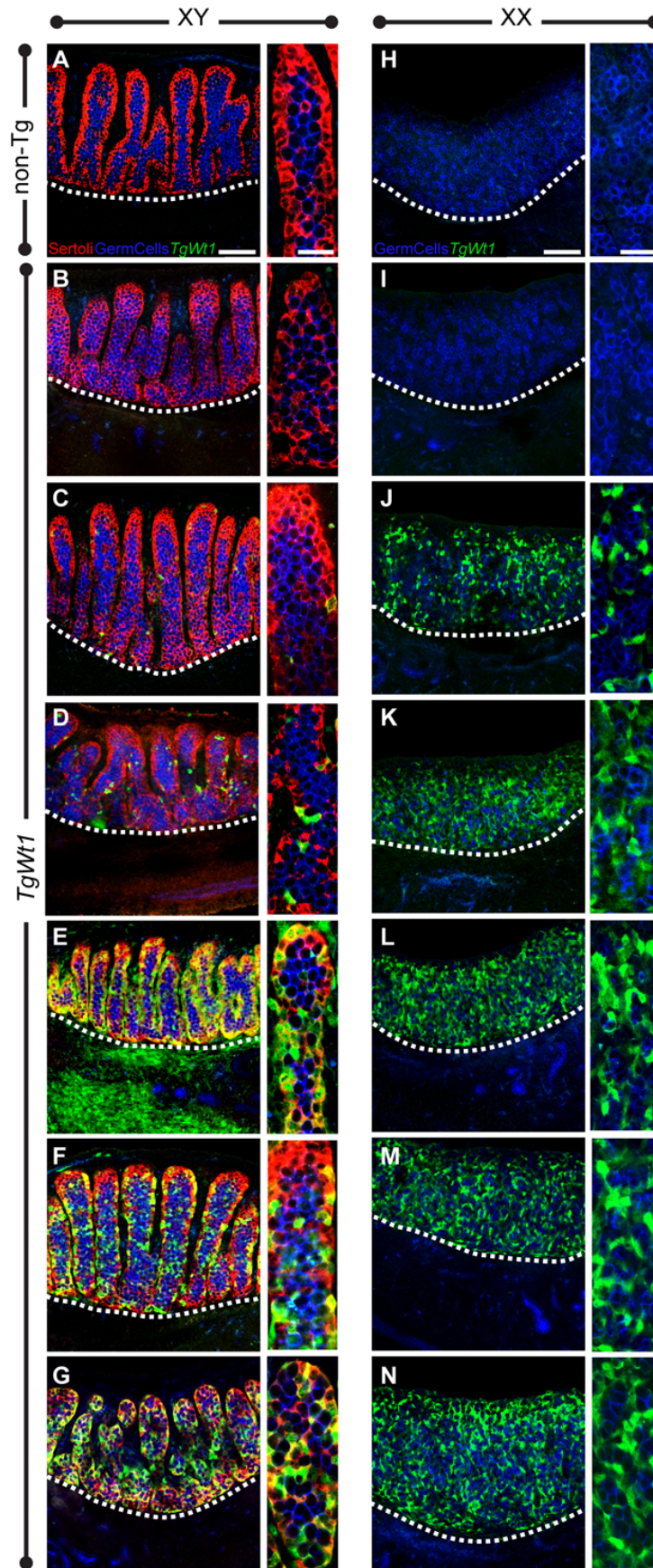
(Jameson 2012).

Figure S5. Transient transgenic analysis identifies a putative *Wt1* enhancer. Embryos were collected at E13.5. The gonad/mesonephros was removed for immunostaining and the remaining embryo was XGAL stained to detect βgal activity (not shown). The gonad, with attached mesonephros, was immunostained with AMH to label Sertoli cells (red), CDH1 to label germ cells (blue) and an antibody to β–galactosidase to detect the *TgWt1* transgene (green). Images were taken by confocal microscopy. A non-transgenic XY (A) and XX (H) sample is shown for comparison. (B-G) Transgenic XY embryos showed a range of expression patterns; however, most had expression highly specific to Sertoli cells. One transgenic XX embryo did not express the transgene (I), while the remaining (J-N) had a pattern of expression similar to WT1 in the gonad, with *TgWt1* expression in ovarian somatic cells. There was no consistent expression in any other cell type throughout the embryo. For each column, confocal images on the left were taken using the 20x objective; the scale bar represents 100 µm. Confocal images on the right were either taken with the 40x objective, or are a 2x zoom of the 20x image; the scale bar represents 12.5 µm.

Table S1

Click here to Download Table S1

Table S2

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Table S3

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