Single-nucleus multiomics reveals the gene-regulatory networks underlying sex

determination of murine primordial germ cells

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Abstract

Accurate specification of female and male germ cells during embryonic development is critical for sexual reproduction. Primordial germ cells (PGCs) are the bipotential precursors of mature gametes that commit to an oogenic or spermatogenic fate in response to sex-determining cues from the fetal gonad. The critical processes required for PGCs to integrate and respond to signals from the somatic environment in gonads are not understood. In this study, we developed the first single-nucleus multiomics map of chromatin accessibility and gene expression during murine PGC development in both XX and XY embryos. Profiling of cell-type specific transcriptomes and regions of open chromatin from the same cell captured the molecular signatures and gene networks underlying PGC sex determination. Joint RNA and ATAC data for single PGCs resolved previously unreported PGC subpopulations and cataloged a multimodal reference atlas of differentiating PGC clusters. We discovered that regulatory element accessibility precedes gene expression during PGC development, suggesting that changes in chromatin accessibility may prime PGC lineage commitment prior to differentiation. Similarly, we found that sexual dimorphism in chromatin accessibility and gene expression increased temporally in PGCs. Combining single-nucleus sequencing data, we computationally mapped the cohort of transcription factors that regulate the expression of sexually dimorphic genes in PGCs. For example, the gene regulatory networks of XX PGCs are enriched for the transcription factors, TFAP2c, TCFL5, GATA2, MGA, NR6A1, TBX4, and ZFX. Sex-specific enrichment of the forkhead-box and POU6 families of transcription factors was also observed in XY PGCs. Finally, we determined the temporal expression patterns of WNT, BMP, and RA signaling during PGC sex determination, and our discovery analyses

43   identified potentially new cell communication pathways between supporting cells and

44   PGCs. Our results illustrate the diversity of factors involved in programming PGCs towards

45   a sex-specific fate.

Introduction

Proper formation of germ cells during embryonic development is crucial for ensuring the production of functional gametes[1]. The embryonic precursors to mature gametes, primordial germ cells (PGCs), are the bipotential stem cells of the germline that give rise to eggs and sperm[1,2]. During embryonic development in mice, PGCs commit to the oogenic or spermatogenic lineage in response to sex-determining cues from the gonadal environment in a process termed PGC sex determination[3]. During sex determination, XX PGCs in the fetal ovary commit to oogenesis by entering meiosis immediately after pre-granulosa cell specification[4]. By contrast, XY PGCs in the fetal testis initiate the spermatogenic program and arrest mitotically in response to signals from Sertoli cells[3,5]. Defects in germ cell differentiation, particularly during fetal life, often lead to reproductive diseases, such as infertility[6] and the formation of germ cell tumors[7]. Thus, it is necessary to enhance our understanding of germ cell development so we can better identify the etiologies of reproductive dysfunction in humans.

PGC sex determination is induced by the sex-specific activation of transcription factors (TFs) and downstream gene networks[8]. In XX PGCs, the retinoic acid (RA)-responsive TFs STRA8 and MEIOSIN and the bone morphogenetic protein (BMP)-responsive TF ZGLP1 are required for entry into meiosis and oogenesis[8-10]. These TFs also initiate the expression of genes related to meiotic processes, including *Rec8* and *Sycp1-3*[8]. In contrast, XY PGCs require the expression of cell cycle inhibitors, such as *Bnc2* and *Cdkn2b*, and the male-specific genes, *Nanos2* and *Dnd1*, to enter mitotic arrest[11-14]. During the transition from PGC to oogonium or gonocyte, both XX and XY PGCs must also lose their bipotential state by downregulating the pluripotency-related genes, *Pou5f1, Nanog,* and

Sox2[8]. Consequently, there are multiple layers of gene regulation required for sex

determination of PGCs.

Beyond the patterns of gene expression in PGCs, relatively little is known about how

signals from the gonadal environment activate the expression of sexually dimorphic TFs

and genes in PGCs. First, the gene regulatory networks, *i.e.* TFs and their predicted target

genes, specific to XX and XY PGCs are not well defined. Second, it remains unclear how the

chromatin environment is temporally regulated during PGC sex determination. Finally,

additional data are needed on the patterns of ligand-receptor expression in gonadal

supporting cells and PGCs. Previous reports have used bulk gene regulation and

expression genomics assays to investigate the transcriptional programs underlying PGC

development[15-18]. However, these assays may not have the resolution or sensitivity

required to detect the transient changes in gene regulation among PGC subpopulations that

are essential to sex determination.

In the present study, we employed the integrative genomics method, combined

single-nucleus transcriptome and chromatin accessibility sequencing, to decipher various

layers of gene regulation during PGC sex determination in mice. We comprehensively

profiled 3,054 XX and XY PGCs at embryonic days (E) E11.5, E12.5, and E13.5, which covers

the developmental time frame from bipotential to sexually differentiated PGCs. Single-

nucleus sequencing enabled the detection of sex-enriched regulatory loci, TFs, and gonadal

cues that may be responsible for initiating gene expression in individual PGCs. We first

systematically examined the molecular signatures of PGC subpopulations to identify the

genes and accessible chromatin regions underpinning the sex-specific fates of PGCs. By

combining our epigenomic and transcriptomic data, we predicted *cis*-regulatory elements

and sex-enriched TFs to construct the gene regulatory networks unique to XX and XY PGCs.

Lastly, we probed the cell-cell communication pathways between supporting cells and

PGCs to nominate potentially new ligand-receptor pairs involved in PGC development. Our

results provide insights into the cell fate decisions underlying gametogenesis and sex

determination of PGCs.

Materials and Methods

Animals

The experiments described herein used homozygous Rosa-tdTomato9 females (JAX

007909)   (B6.Cg-Gt(ROSA)26Sor<tm9(CAG-tdTomato)Hze>/J)   crossed   to

homozygous Nr5a1-cre mice (B6D2-Tg(Nr5a1-cre)2Klp), provided by the late Dr. Keith

Parker[19]. Female mice were timed-mated and the day of detection of vaginal plug was

considered embryonic day (E) E0.5. Mice were housed on a 12 h light:dark cycle,

temperature range 70–74 °F and relative humidity range from 40 to 50%. All animal

studies were conducted in accordance with the NIH Guide for the Care and Use of

Laboratory Animals and approved by the National Institute of Environmental Health

Sciences (NIEHS) Animal Care and Use Committee.

Single-nucleus RNA-sequencing and ATAC-sequencing

Whole ovaries and testes were collected at E11.5, E12.5, and E13.5. The sexes of the

embryos were determined using amnion staining, as previously published[20], to identify the

presence (XX) or absence (XY) of the Barr body and confirmed with PCR using primers that

recognize the Y chromosome. Tomato fluorescence was used to facilitate the dissection of

the gonad away from the mesonephros, which was done using needles. Isolated fetal mouse

gonads were isolated in PBS, and pairs of gonads or groups pooled between fetuses of the

same sex and age were immediately frozen in liquid nitrogen for storage. Nuclei isolation

was performed using the 10X Genomics Chromium Nuclei Isolation Kit (catalog #1000494)

following the manufacturer's protocol. The total numbers of gonads used per sex and age

are listed in **Supplementary Table 1**. The 10X Genomics Chromium Next GEM Single Cell

Multiome ATAC + Gene Expression Library Preparation Kit (catalog #1000284) was used

to generate a 10X barcoded library of mRNA and transposed DNA from individual nuclei.

The 10X barcoded single-nuclei RNA and ATAC-seq libraries were obtained from 8,687

average (1,555-20,000) nuclei per sample and sequenced with Illumina NOVAseq to a

minimum sequencing depth of 129,120,442 raw reads and 22,406 read pairs/nucleus

(**Supplementary Table 1**). Two independent technical replicates of multiome sequencing

were performed for each sex by embryonic stage combination.

Supplementary Table 1. Sequencing and QC statistics for single-nucleus multiome

libraries of E11.5-E13.5 XX and XY gonads.

Sample	Pooled Paired Gonads (Number)	Raw Reads	Barcode	Mapped	Saturation	Cells	Reads/cell	Median UMI/cell	Median genes/cell
E11.5 F_R1	19	448,115,792	93%	91%	38%	20,000	22,406	3,665	2,030
E11.5 F_R2	11	812,813,546	94%	92%	68%	10,008	81,216	8,184	2,798
E11.5 M_R1	18	193,672,880	93%	94%	35%	8,080	23,969	2,790	1,708
E11.5 M_R2	12	719,219,983	94%	91%	64%	4,960	145,326	5,934	3,512
E12.5 F_R1	12	498,266,895	93%	94%	43%	18,696	26,651	4,620	2,422
E12.5 F_R2	11	654,449,771	94%	91%	66%	4,949	131,946	8,351	3,504
E12.5 M_R1	8	425,422,729	93%	91%	44%	14,897	28,558	5,268	2,682
E12.5 M_R2	10	951,070,062	94%	92%	74%	5,864	162,188	9,194	3,446
E13.5 F_R1	3	135,990,358	95%	92%	86%	1,555	83,036	3,608	2,121
E13.5 F_R2	5	398,709,188	95%	92%	73%	6,369	74,137	6,013	2,831
E13.5 M_R1	3	129,120,442	94%	94%	82%	2,040	66,662	5,132	2,642
E13.5 M_R2	7	472,180,945	94%	91%	71%	6,597	60,438	6,546	2,969

Data preprocessing for single-nucleus RNA-seq and ATAC-seq libraries

CellRanger[21,22] (v3.0) was used for count, alignment, filtering, and cell barcode and UMI

counting (**Supplementary Table 1**). Barcode swapping correction was performed for all

libraries[23]. FASTQ files of the corrected cell count matrices were generated and the data

were analyzed using *Seurat*[24] (v. 4.3.0) and *Signac*[25] (v. 1.6.0) on R (v. 4.1.0).

snRNA-seq data preprocessing. Doublets within each dataset were removed with the

scDblFinder[26] (v. 1.8.0) package using default methods. Ambient RNA was removed using

the *celda/decontX*[27] (v. 1.12.0) package, with the maximum iterations of the EM algorithm

set at 100. Datasets of gonadal cell populations from each embryonic stage and sex were

combined into one *Seurat* object using the "merge" function. As batch effect was not

observed in our dataset, we did not perform further integration or batch correction. Cells

with nCount_RNA > 1000 & nCount_RNA < 25,000& percent.mt <25 were retained for

downstream analyses (**Supplementary Figure 1a**). The data was normalized using the

"SCTransform" function in *Seurat*.

snATAC-seq data preprocessing. snATAC-seq peak calls from *CellRanger* were used to

generate one combined peak set and cells with peak size > 20 bp & peak size < 10,000 bp

were retained for downstream analyses. Fragment objects were created for each library,

combined with the respective *Seurat* object, and analyzed using the *Signac* package. A

subset of the snATAC-seq data was generated using the following cutoffs: nCount_ATAC <

100,000 & nCount_ATAC > 1000 & nucleosome_signal < 2 & TSS.enrichment > 1

(**Supplementary Figure 1b-d**). Analysis of the snATAC-seq peak feature distribution

demonstrated that most peaks occurred in distal intergenic or promoter regions of the

genome (**Supplementary Figure 1e**).

Sex filtering. To ensure that the correct sex was analyzed in all downstream analyses, we

applied computational filters to confirm the sex of the cells in each dataset. Cells were

filtered based on Y-linked gene expression and fragments mapped to the Y chromosome

(chrY). The *UCell* package[28] (v. 2.0.1) was used to create a module score of the Y-linked

genes *Kdm5d, Eif2s3y, Uty,* and *Ddx3y*. Module scores of E11.5 and E12.5 cells above one

standard deviation of the mean module score of all XY E13.5 cells were determined to be

chromosomally male. Module scores of E11.5 and E12.5 cells below one standard deviation

of the mean module score of all XX E13.5 cells were determined to be chromosomally

female. Next, we examined the peaks called on chrY outside of the pseudoautosomal region.

In total, 21 peaks were identified between chrY 1-90,000,000 bp. Cells with 0 fragment

counts within our defined chrY peak region and chrY gene expression module score < 1

standard deviation were removed from the male datasets. Cells with > 1 fragment counts

within our defined chrY peak region and chrY gene expression module score > 1 standard

deviation were removed from the female datasets.

Cell type identification

To identify the cellular origin of the snRNA-seq clusters, principal component analysis

(PCA) was run on the scaled data and visualization was performed using UMAP with dims =

18 and resolution = 0.3. Primordial germ cell (PGC) cluster identification was performed

using the established germ cell markers *Ddx4* and *Pou5f1*[29]. To remove potential PGC and

gonadal somatic cell doublets, only cells with RNA count < 1 for the following genes were

retained as PGCs: the granulosa cell marker genes *Foxl2* and *Runx1*[30]; Sertoli cell marker

Sox9[30]; Leydig cell marker *Insl3*[30]; stromal cell marker *Wt1*[31]; endothelial cell marker

Plvap[32]; immune-related cell marker *Mafb*[33]; interstitial cell markers *Pdgfra* and *Nr2f2*[30];

supporting cell marker *Tspan8*[34]; and epithelial cell marker *Krt19*[35]. Somatic supporting

cell cluster identification was performed using the established female and male support cell

markers *Runx1* and *Sox9*, respectively. To remove potential gonadal supporting cell

doublets formed with germ cells or interstitial cells, only cells with RNA count > 1 for *Wt1*

and RNA count < 1 for the following genes were retained as support cells: the germ cell

marker genes *Ddx4* and *Pou5f1*; endothelial cell markers *Plvap* and *Pecam1*[36]; and

interstitial cell markers *Pdgfra* and *Nr2f2*.

183 Clustering and pseudotime trajectory of primordial germ cells

snRNA-seq reclustering of PGCs was done with PCA on scaled PGC RNA data and

visualization was performed using UMAP with dimensions (dims) = 18 and resolution =

0.3. snATAC-seq clustering of PGCs was done first by calling peaks using *MACS2*[37] (v.

2.2.7.1). Peaks on nonstandard chromosomes were removed using the

"keepStandardChromosomes" function in *Signac* with the option 'pruning.mode = "coarse"'

and peaks in mm10 genomic blacklist regions were removed. Counts in each peak were

determined using *Signac* functionalities. The data was normalized by latent semantic

indexing (LSI) using the "RunTFIDF", "FindTopFeatures", and "RunSVD" functions in *Signac*.

Visualization was performed using UMAP with dims = 2:30, reduction = 'lsi', and algorithm

= 3. The joint neighbor graph or joint UMAP was generated using the

"FindMultiModalNeighbors" function in Signac and reduction.name = "wnn.umap". A Seurat

object from the clustered PGCs was used as input for pseudotime analysis with Monocle3 to

determine the differentiation trajectory of PGCs using both female and male E11.5 PGCs as

time '0' in pseudotime.

198 Identification of differentially expressed genes and differentially accessible peaks

Differentially expressed genes (DEGs) in each cluster were determined using the

"FindAllMarkers" function in *Seurat* based on the Wilcoxon Rank Sum Test. DEGs between

female and male PGCs at each embryonic stage were identified with the "FindMarkers"

function in *Seurat* with min.pct = 0.25 and logfc.threshold = 0.25. Differentially accessible

peaks (DAPs) between female and male PGCs were identified with the "FindMarkers"

function in *Signac* with min.pct = 0.001 and logfc.threshold = 0.1.

Correlation of peak accessibility with gene expression

snATAC-seq peaks were linked to gene expression, as imputed from the snRNA-seq data,

using the "LinkPeaks" function in *Signac*. Peaks linked to DE genes for each sex and

embryonic stage were then subsetted out for further downstream analyses. Plots showing

peak-to-gene linkages were generated using the "CoveragePlot" function in *Signac*.

Motif enrichment analysis of transcription factor binding sites

DNA sequence motif analysis of peaks linked to DE genes was performed using the

"FindMotifs" function in *Signac*. Background peaks were selected to match the GC content

in the peak set by using the "AccessiblePeaks," "GetAssayData", and "MatchRegionStats"

functions in *Signac. MotifScan*[38] (v. 1.3.0) was used to determine the genomic position of

linked peaks to DE genes to identify predicted target genes of transcription factors. Per-cell

motif activity was computed using the "RunChromVAR" function in *Signac*.

Module score calculation for gene expression

Module scores of gene expression were calculated using the "AddModuleScore" function in

Seurat. Module scores were plotted on UMAPs using the "FeaturePlot" function in *Seurat*.

Peak feature distribution

Pie charts of peak feature distribution were generated with the R package *ChIPseeker*[39,40]

(v. 1.38.0). Peaks were annotated with the "annotatePeak" function with options

'tssRegion= c(-3000,3000)', 'TxDb=TxDb.Mmusculus.UCSC.mm10.knownGene', and

'annoDb="org.Mm.eg.db". Pie charts were plotted with the function "plotAnnoPie".

GO annotation and enrichment

The GO enrichment analyses were carried out using the R package *clusterProfiler*[41] (v. 4.0.5) with the "enrichGO" function. The Benjamini-Hochberg method (a = 0.05) was used to control for False Discovery Rate. GO annotation enrichment for biological processes were plotted using the "dotplot" or "cnetplot" function of the *Enrichplot*[42] (v1.12.2) R package.

CistromeDB regulatory potential score

The CistromeDB Regulatory Potential Score[43,44] was used to identify potential regulators of *Bnc2* and *Stra8*. The toolkit (http://dbtoolkit.cistrome.org) was used for these analyses by selecting Mouse mm10 as 'Species', transcription factor/chromatin regulator as 'Data Type in Cistrome', and 10 kb as 'The half-decay distance to transcription start site.'

Enrichment of signaling pathways between gonadal support cells and primordial germ cells

CellphoneDB[45] (v. 5.0.0) was used to predict paired ligand-receptor pairs for cell-cell communication between gonadal supporting cells and PGCs. Normalized snRNA-seq counts were used as input data. Significant ligand-receptor pairs were determined independently between gonadal supporting cells and PGCs for each embryonic stage and sex. The statistical analysis (method 2) of *CellphoneDB* and the 'statistical_analysis_method' function in *CellphoneDB* were used to identify significantly enriched signaling pathways.

Immunofluorescence

Gonads were collected from E13.5 XX and XY embryos and fixed in 4% PFA for 1h at room temperature. Gonads were then washed in PBS and stored at 4 °C until embedding in paraffin and sectioned at 5 μm thickness. Following deparaffinization, sections were

rehydrated in decreasing alcohol concentration gradients. Antigen retrieval was performed

with 0.1 mM citric acid (Vector Labs) for 20 min in the microwave at 10% power and then

cooled to room temperature. Samples were blocked in PBS-TRITON X-100, 0.1% solution

with 5% normal donkey serum for 1h. Samples were incubated with primary antibodies

diluted in blocking buffer (PBS-TRITON X-100, 0.1% solution with 5% normal donkey

serum) at 4 °C overnight. Samples were then washed with PBS-TRITON X-100, 0.1%

solution and incubated with secondary antibodies diluted in blocking buffer at room

temperature for 1h. The slides were washed with PBS-TRITON X-100, 0.1% solution 3x and

incubated with the Vector TrueView Autofluorescence Quenching Kit (cat. # SP-8400) for 2

min. Following incubation with the quenching kit, the slides were washed with PBS-

TRITON X-100, 0.1% solution 2x, PBS 1x, and incubated with DAPI (Invitrogen cat. #

D1306). The slides were then mounted with slide mounting media (Vectashield Vibrance

Antifade Mounting Medium TrueView Kit SP-8400). Primary antibodies used included:

Anti-TRA98 (1:500; rat, BioAcademia cat. # 73-003), Anti-NR2F2 (1:300, mouse, R&D

Systems cat. # PP-H7147-00), Anti-PORCN (1:200, rabbit, ThermoFisher cat. # PA5-43423),

and Anti-AP-2γ (TFAP2c) (1:200, mouse, Santa Cruz cat. # sc-12762). Secondary antibodies

were used at 1:200 dilution (Invitrogen/Life Technology): Donkey anti-rat Alexa 568

(A78946), Donkey anti-mouse Alexa 647 (A31571), and Donkey anti-rabbit Alexa 488

(A21206). Imaging for sections was performed on a Zeiss LSM 900 confocal microscope

using Zen software. Brightness and contrast of images were adjusted using ImageJ

(National Institutes of Health, USA).

Statistical analyses

Libraries were prepared from 3-19 pairs of gonads and two technical replicates per

embryonic stage and sex (**Supplementary Table 1**). Statistical analyses were considered

significant if $p < 0.05$.

Results

Combined multiome analysis of single-nucleus gene expression and chromatin accessibility of mouse PGCs

To understand how intrinsic transcription networks inside PGCs and external cues from the gonadal environment control PGC sex determination, we performed combined snRNA-seq and snATAC-seq (10x Genomics Multiome) for all cell populations in mouse fetal gonads. This approach allowed us to obtain paired gene expression and chromatin status from the same cell for all cell populations from female (XX) and male (XY) gonads. Whole gonads from Nr5a1-cre x Rosa-tdTomato9 embryos were collected at embryonic days (E) E11.5, E12.5, and E13.5, which encompass the developmental time frame of sex determination-initiated (E11.5), sexually differentiating (E12.5), and morphologically dimorphic (E13.5) gonads. Sequencing libraries of gonads for each sex and embryonic day were generated from two independent technical replicates for an average 8,687 nuclei/sample with a sequencing depth of ~75,544 reads/nucleus and ~5,755 UMIs/nucleus. Based on the well-established germ cell markers *Ddx4*[29] and *Pou5f1*[29], we identified 3,054 germ cells. Somatic supporting cell populations were identified using *Runx1* expression as a marker of pregranulosa cells in our female datasets and *Sox9* expression as a marker of Sertoli cells in our male datasets[30]. A combined 22,382 XX and XY supporting cells were identified. Together, these data allowed us to determine the clustering patterns of differentiating PGCs, identify regulatory loci and transcription factors (TFs) enriched in PGCs, and find previously unreported interactions between PGCs and gonadal supporting cells (Fig. 1a).

We first asked whether single-nucleus gene expression and chromatin accessibility information can quantify the similarity or differences within PGC populations. We clustered single PGC nuclei in low-dimensional space based on their snRNA-seq (**Fig. 1b-d**) or snATAC-seq (**Fig. 1e-g**) data using Uniform Manifold Approximation and Projection (UMAP) for dimensional reduction. This graph-based clustering algorithm unbiasedly separated and clustered individual cells with similar transcriptomic profiles[46]. Such analyses revealed that the transcriptomes of E11.5 XX and XY PGCs overlap extensively, whereas E12.5 and E13.5 PGCs principally separated according to their sex (**Fig. 1b**). In addition, we uncovered eight broader clusters of PGCs at a resolution that captured PGC subpopulations (**Fig. 1c**). We determined the cell type composition of the snRNA-seq transcriptome-based clusters by plotting the percentage of PGCs at each embryonic stage and sex (*x*-axis) in each of the 8 transcriptome-based clusters (*y*-axis) (**Fig. 1d**). First, E11.5 and E12.5 XX PGCs primarily consisted of clusters 0, 1, and 4 (**Fig. 1c-d**). At E13.5, XX PGCs separated into three main clusters, 4, 5, and 6, and had significantly higher expression of the synaptonemal complex component and meiotic marker *Sycp1*[47] and the chromatin modifier *Hdac9*[48] (**Fig. 1c-d; Supplementary Figure 2**). In XY gonads, PGCs at E11.5 grouped together into three clusters, 0, 1, and 7 (**Fig. 1d**). At E12.5-E13.5, XY PGCs converged onto a single distinct population (cluster 7), indicating less transcriptional diversity among E12.5-E13.5 XY PGCs when compared to E12.5-E13.5 XX PGCs (**Fig. 1d**). Furthermore, we found that E12.5-E13.5 XY PGCs clustered separately from other PGC populations due to elevated expression of *Bnc2*, which is required for mitotic arrest in PGCs[11], and the forkhead-box TF *Foxp1*[49] (**Supplementary Figure 2**). Finally, a small proportion of all PGC populations (E11.5-E13.5 XX and XY PGCs) were detected in clusters

2 and 3, and displayed enriched expression of ribosome biogenesis genes, the

mitochondrial leucyl-tRNA synthetase *Lars2*[50], and the DNA repair protein *Mgmt*[51] (**Fig. 1d;**

Supplementary Figure 2). Together, these data indicate that the transcriptomic profiles of

PGCs are largely homogenous within each sex at E11.5. Subsequently, XX PGCs maintain

their transcriptional heterogeneity at E12.5-E13.5, whereas XY PGCs transcriptionally

converge on a single identity beginning at E12.5.

The snRNA-seq clustering revealed transcriptomic characteristics of PGCs. Next, we

performed unbiased clustering of PGCs according to their aggregate profiles of snATAC-seq

peaks (210,666 total peaks) to detect the patterns of chromatin accessibility associated

with PGC development (**Fig. 1e and 1f**). The snATAC-seq-based clustering of PGCs showed

that the chromatin status of E11.5 XX and XY PGCs was highly similar (**Fig. 1e**). By contrast,

E12.5-E13.5 XX and XY PGCs formed distinct groupings at opposite poles of the UMAP (**Fig.**

1e). Unbiased graph-based clustering of PGCs uncovered 9 clusters of PGCs with unique

stage- and sex-specific snATAC-seq peak profiles (**Fig. 1f**). First, E11.5-E12.5 XX and XY

PGCs together predominantly consisted of accessibility-based clusters 0, 1, and 2,

indicating that these germ cells share a highly similar chromatin profile prior to

differentiation (**Fig. 1f and g**). Second, E13.5 XX PGCs were separated into clusters 3, 4, and

5 (**Fig. 1f and g**), which may be due to differences in meiotic entry or progression within

these populations of germ cells. Third, E12.5-E13.5 XY PGCs comprised snATAC-seq

clusters 6 and 7, suggesting that XY PGCs may adopt a sex-specific chromatin status

beginning at E12.5 (**Fig. 1f and g**). Finally, cluster 8, which contained PGCs from both sexes

at each stage, was separate from all other accessibility-based clusters (**Fig. 1f and g**). Taken

together, our findings indicate that the chromatin accessibility profiles of E11.5 PGCs and

several E12.5 PGC subpopulations do not show a sex bias, whereas E13.5 XX and XY PGCs

clustered by sex.

The multiome datasets provided us the opportunity to define PGC identities based

on a more biologically relevant status at the combined transcriptomic and chromatin

accessibility levels. Specifically, we integrated gene expression and chromatin accessibility

data within a single PGC to obtain a joint, or multi-modal, definition of PGC identity (**Fig. 1h**

and i). Computational integration methods allowed us to co-visualize the variation in both

the snRNA-seq and snATAC-seq profiles of PGCs on a single UMAP[52]. Simultaneously

profiling cell-type specific transcriptomes and regions of open chromatin for single PGCs

resolved the sex-specific trajectories of PGCs with much greater resolution (**Fig. 1h and i**).

In fact, we observed a strong demarcation between XX and XY PGCs beginning at E12.5

with very little to no overlap between XX and XY PGCs at E12.5-E13.5 (**Fig. 1h-j**). Given that

we detected the clearest separation of PGCs when clustering with multi-modal data, we

analyzed the trajectory of transcriptomic changes (also known as pseudotime analysis in

Monocle[53]) among PGCs overlaid on the joint UMAP (**Fig. 1h**). With E11.5 XX and XY PGCs

chosen as the starting point (0) of the trajectory analysis, we observed a distinct branching

or 'decision' point (1) between XX and XY PGCs at E12.5 (**Fig. 1h**). Additionally, our

unbiased analysis identified two terminal states (2) of PGCs at the E13.5 XX PGC cluster and

E13.5 XY PGC cluster, providing additional confirmation that our data accurately reflect the

status of PGC sex determination (**Fig. 1h**). We also observed a closed, or circular, branch of

the trajectory path among E12.5 XX PGCs (**Fig. 1h**), which may arise from the

heterogeneous transcriptomes of female PGCs entering meiosis in an asynchronous

manner[54]. These data demonstrate that our approaches enable us to not only visualize the

developmental trajectories of PGCs that are clustered with multi-modal data, but also

empirically trace their sex-specific fates based on changes in gene regulation.

Clustering of PGCs using combined snRNA- and snATAC-seq data identified eleven

distinct groups of PGCs and yielded enhanced cluster resolution of PGC subpopulations

(**Fig. 1i**). This analysis resulted in clusters of PGCs that were predominantly sex-specific

and enabled the detection of subpopulations of each PGC type (**Fig. 1i**). To better

understand the population structure of individual clusters, we plotted the percentage of

each PGC type (*x*-axis) for each cluster identified in **Fig. 1i** (*y*-axis) (**Fig. 1j**). First, E11.5 XX

PGCs clustered into one main population, cluster 1, and later diverged into six

subpopulations, clusters 0 through 5 at E12.5 (**Fig. 1i-j**). Clusters 0 - 2 were more similar to

E11.5 PGCs, whereas clusters 3 – 5 were composed of E12.5 XX PGCs that were

developmentally closer to E13.5 XX PGCs (**Fig. 1i-j**). Finally, we found that E13.5 XX PGCs

converged onto either one of two developmental fates in clusters 6 and 7, with cluster 7

exhibiting a large spread along the UMAP2 axis (**Fig. 1i-j**). These findings indicate that

E12.5-E13.5 XX PGCs may require extensive chromatin remodeling and transcriptional

reprogramming to adopt the oogenic fate. By comparison, XY PGCs grouped into clusters 0

and 1 at E11.5 and appeared to converge on a single developmental fate at later stages,

with E12.5 XY PGCs comprising clusters 8 and 9 and E13.5 XY PGCs belonging to cluster 10

(**Fig. 1i-j**). Given that neither expression- nor accessibility-based clustering alone resolved

the granularity of PGC populations to the level described here, these data underscore the

empirical power of simultaneously measuring multiple modalities from the same cell to

define the developmental fate of PGCs.

Molecular characterization of genes known for their roles in XY PGC differentiation

We next asked whether our multiomics approaches could gain insight into the regulatory status of genes that are known to be essential for PGC sex determination. We first investigated the patterns of gene expression and chromatin accessibility of male functional genes, *i.e.* those that have been shown to be functionally important for XY PGC development (**Fig. 2**). We found that genes involved in XY PGC development (*i.e., Rb1, Rbl2, Cdkn1b, Cdkn2b, Bnc2, Cnot1, Dnd1,* and *Nanos2*)[11-14,55] showed increasing levels of expression in XY PGCs from E11.5 to E13.5, whereas the expression levels of *Cnot1* and *Nanos3* peaked at E12.5 and E11.5, respectively (**Fig. 2a**). Notable XY-enrichment of *Bnc2* expression and moderately elevated expression of *Rb1*, *Rbl1*, *Cdkn1b*, *Cdkn2b*, *Nanos2*, and *Nanos3* were observed in XY PGCs compared to XX PGCs (**Fig. 2a**). However, expression of *Cnot1* and *Dnd1* showed an XX biased expression pattern (**Fig. 2a**). Overall, these data indicate that the majority of male functional genes show a modest increase in expression levels in XY PGCs over XX PGCs. Among these genes, *Bnc2* expression may serve as a definitive marker of XY PGCs at E13.5.

To identify potential mechanisms regulating the expression of XY PGC functional genes, we searched for candidate transcription factors (TFs) with XY-enriched gene expression. We first linked snATAC-seq peaks to XY PGC functional genes (listed previously), hereafter termed peak-to-gene linkages, in XY PGCs. Next, we performed a TF motif enrichment analysis on significant peak-to-gene linkages to identify potential TFs. Finally, we compared the expression levels of potential TFs between XX and XY PGCs to identify TFs with XY-enriched gene expression. This strategy uncovered the Krüppel-like zinc finger family member ZKSCAN5 (Zinc finger with KRAB and SCAN domains 5) as a

potential regulator of XY PGC functional genes (**Fig. 2a**). *Zkscan5* showed XY biased

expression and the level of *Zkscan5* expression increased from E11.5 to E13.5 in XY PGCs

(**Fig. 2a**). In addition, the ZKSCAN5 motif was not enriched in XX PGCs. ZKSCAN5 is not only

highly expressed in the adult testis[56] but is also proposed to regulate cellular proliferation

and growth[57]. Thus, although little is known about the function of ZKSCAN5, we

hypothesize ZKSCAN5 may possess a regulatory role during male germ cell development.

These data highlight the strength of single-nucleus multiomics in discovering candidate

factors that may regulate the expression of a cohort of developmental genes and,

consequently, direct cell fate decisions.

Among the functionally important PGC genes, *Bnc2* expression was most strongly

enriched in XY PGCs. We therefore investigated the regulation of *Bnc2* at the chromatin

level as an example to demonstrate the utility of combined multiome datasets. The

chromatin accessibility of the *Bnc2* promoter was highest in XY PGCs and increased

temporally from E11.5 to E13.5 (**Fig. 2b**). Furthermore, the level of chromatin accessibility

at the *Bnc2* locus was positively correlated with *Bnc2* expression in XY PGCs (**Fig. 2b-c**). We

then applied the Cistrome Data Browser (Cistrome DB)[44] to identify putative regulators of

Bnc2 expression based on transcription factor binding motifs detected within 10 kb of the

Bnc2 promoter (**Fig. 2d**). The top candidate regulators of the *Bnc2* locus were determined

according to a regulatory potential score derived from a combination of TF binding motif

information and previously published genomics data (*i.e.* ChIP-seq, DNase-seq, and ATAC-

seq) (**Fig. 2d**). Of the top candidate regulators from the Cistrome DB analysis, expression of

Ctcf, *Nrf1*, *Polr2a*, and *Brd4* was enriched in XY PGCs at E13.5 (**Fig. 2e**). In particular, BRD4

is known to activate transcription elongation by recruiting kinases to the largest RNA

polymerase II (Pol II) subunit, POLR2A, thereby releasing Pol II from its promoter-proximal

bound state[58]. Taken together, these findings provide insight into potential XY-specific

regulatory mechanisms at the *Bnc2* locus in PGCs.

Characterization of genes known for their roles in XX PGC development

We next performed the same analyses of XX PGC functional genes, *i.e.* those that

have been shown to be functionally important for XX PGC development (**Fig. 3a**). Overall,

as expected, we found that E13.5 XX PGCs expressed genes involved in XX germ cell

development (*i.e., Rec8, Sycp2, Sycp3, Ctnnb1, Meioc, Rnf2, Stra8, Ythdc2,* and *Zglp1*)[8]

substantially higher than E13.5 XY PGCs (**Fig. 3a**). Moreover, *Rnf2* and *Zglp1* were strongly

expressed at E12.5 in XX PGCs, and their expression occurred immediately prior to the

activation of all other genes in the female cohort (**Fig. 3a**). Although *Zglp1* was expressed in

fewer XX PGCs than *Rnf2*, *Zglp1* expression increased from E12.5 to E13.5 whereas *Rnf2*

expression decreases (**Fig. 3a**). These data support previous studies that *Rnf2* and *Zglp1*

control the timing of sexual differentiation of XX PGCs[10,59]. By comparison, the expression

of *Ctnnb1* diminished in both XX and XY PGCs at E12.5, as expected given that negative

regulation of WNT/β-catenin is required for XX PGCs to enter meiosis[60] (**Fig. 3a**). In sum,

these results provide insight into the timing, expression levels, and pervasiveness of female

gene activation in XX PGC populations.

We next identified potential regulators of XX PGC functional genes using the same

approach for XY PGCs (**Fig. 2**). Our analysis identified significant enrichment of the binding

motif for DMRT1 in the regulatory elements linked to XX PGC functional genes (**Fig. 3a**).

DMRT1 has been shown to control *Stra8* expression in a sex-specific manner, such that

Stra8 is activated in the fetal ovary and repressed in the fetal testis[61]. Our snRNA-seq data

also demonstrated *Dmrt1* expression in both XX and XY PGCs (**Fig. 3a**), which is consistent

with previous findings[61].

Since *Stra8* plays an essential role in controlling meiotic entry[8], we next sought to

further understand the gene regulatory mechanisms underlying *Stra8* activation. First, we

not only found that accessibility of the *Stra8* promoter was highest in XX PGCs, but also that

Stra8 chromatin accessibility was positively correlated with *Stra8* gene expression (**Fig.**

3b-c). Second, the population of E13.5 XX PGCs displaying the strongest *Stra8* expression

levels corresponded to the same population of XX PGCs with the highest module score of

early meiotic prophase I genes (**Fig. 3c; Supplementary Fig. 3a-b**). Next, to determine

putative regulators of *Stra8* expression, we identified enriched TF binding motifs within 10

kb of the *Stra8* promoter using Cistrome DB[44] (**Fig. 3d**). Of the factors with the highest

Cistrome DB Regulatory Potential Score, we observed XX-enriched expression of *Rxra,*

H2afy, Tcf12, Rad21, Smc3, and *Smad3* (**Fig. 3e**). Several of these factors, such as RAD21 and

SMC3, are members of the cohesin complex, which serves both a structural and gene

regulatory role during meiotic prophase I[62,63]. Moreover, RXRA has been shown to

positively influence *Stra8* expression levels during oocyte development in the mouse[64].

Taken together, these data provide a proof-of-principle example for the application of

single-nucleus multiomics in identifying putative regulators of germ cell fate.

Expression and *in silico* chromatin binding of retinoic acid receptors in PGCs

Since recent reports have demonstrated that meiosis occurs normally in the fetal

ovary of mice lacking key members of the retinoic acid (RA) signaling pathway[65], we next

asked whether we could detect any evidence for gene regulation by RA receptors in PGCs. Previous studies have demonstrated that RA from the mesonephros induces meiosis in the fetal ovary whereas in the fetal testis, RA is degraded by the enzyme CYP26B1, therefore preventing PGC entry into meiosis[66,67]. However, this model was challenged by the findings that meiosis, and consequently oogenesis, still occurs in XX gonads of mice lacking the RA-synthesizing enzyme, ALDH1, or all RA receptors[65,68]. Nevertheless, *Stra8* expression was significantly reduced in E13.5 fetal ovaries without *Aldh1a1-3* or all RA receptors[65,68], thus indicating that *Stra8* expression is likely controlled by the RA signaling pathway, in addition to others. We therefore investigated the expression and *in silico* chromatin binding patterns of members of the RA signaling pathway (*i.e. Rara, Rarb, Rarg, Rxra, Rxrb, Rxrg, Crabp1,* and *Crabp2*)[69] (**Supplementary Figure 4a-b**). We found that expression of the retinoic acid receptor beta, *Rarb*, and cellular retinoic acid binding protein 1, *Crabp1*, were enriched in XX PGCs beginning at E12.5 and continuing to E13.5 (**Supplementary Figure 4a-b**). Similarly, E13.5 XX PGCs showed enriched expression of the retinoid X receptor alpha, *Rxra*, and cellular retinoic acid binding protein 2, *Crabp2* (**Supplementary Figure 4a-b**). By contrast, the expression of *Rara, Rarg,* and *Rxrb* was enriched in XY PGCs, and *Rxrg* was lowly expressed in both XX and XY PGCs (**Supplementary Figure 4a-b**). We also observed significant enrichment of the 'motif activity score', an *in silico* measurement of the contribution of a specific motif to gene regulation[70], of all RA receptors in E13.5 XX PGCs (**Supplementary Figure 4c-d**). These data indicate that the RA receptors display strong evidence of *in silico* chromatin binding in E13.5 XX PGCs but not E13.5 XY PGCs. Furthermore, the binding motifs for the RA receptors were predominantly located in E13.5 XX PGC chromatin peaks that were distal intergenic, intronic, or within promoters

(**Supplementary Figure 4e;** motifs for RARB and RXRA provided as examples). These data

indicate that the binding motifs for the RA receptors are predominantly enriched in both

distal and proximal regulatory elements in E13.5 XX PGCs. The RA receptor motifs were

also statistically linked to genes involved in 'response to retinoic acid', 'negative regulation

of cell cycle', and 'meiotic cell cycle' (**Supplementary Figure 4f;** motifs for RARB and RXRA

provided as examples). Finally, when we searched for the presence of RA receptor motifs in

peaks linked to genes related to meiosis and female sex determination, we found that *Stra8,*

Rec8, Rnf2, Sycp1, Sycp2, Ccnb3, and *Zglp1* contain the RA receptor motifs in their

regulatory sequences (**Supplementary Figure 4g**). Together, these *in silico* experiments

indicate that the RA signaling pathway directly regulates the expression of several meiosis-

related genes.

Discovery of differentially expressed genes and differentially accessible regulatory

loci underlying sex determination of PGCs

We further investigated our multiome dataset to discover genes and *cis*-regulatory

elements enriched in PGCs in a sex-specific manner. First, we analyzed which annotated

protein-coding genes showed transcriptional changes between XX and XY PGCs at each

embryonic stage. Differential expression analysis (log_2fold-change > 0.25) of snRNA-seq

data identified 87, 301, and 585 XX PGC-enriched genes and 75, 344, and 544 XY PGC

enriched genes at E11.5, E12.5, and E13.5, respectively (False Discovery Rate-adjusted *p*-

value < 0.05; **Fig. 4a**). The number of differentially expressed genes (DEGs) increased by

~3.5-fold during the transition from E11.5 to E12.5 PGCs and by 1.75-fold during the

transition from E12.5 to E13.5 PGCs (**Fig. 4a**). These findings demonstrate that the number

of DEGs steadily increased over developmental time in both XX and XY PGCs.

An in-depth analysis of the DEGs between XX and XY germ cells identified sex-

enriched genes for PGCs at each embryonic stage (**Supplementary Figure 5a**). For

example, we found elevated expression of the WNT protein regulator *Porcn*[71], the beta-

catenin binding protein *Grip1*[72], and the growth regulator *Phlda2*[73] in XX PGCs

(**Supplementary Figure 5a**). These observations are consistent with the fact that WNT/β-

catenin signaling is involved in specifying the female fate of PGCs[8]. We also identified XY

enriched DEGs, which included the histone demethylase *Kdm5d*[74], the regulator of

pluripotency *Dppa5*[75], and the forkhead transcription factor *Foxp1*[76] (**Supplementary**

Figure 5a). Gene ontology (GO) analyses of DEGs revealed several biological processes that

show sex-enriched expression patterns (**Supplementary Figure 5b**). For example, the

DEGs identified in XX PGCs were assigned to the 'meiotic chromosome organization', 'sister

chromatid cohesion', and 'WNT signaling pathway' gene ontologies (**Supplementary**

Figure 5b). The DEGs identified in XY PGCs were related to 'mitotic cell cycle', 'TGFβ

signaling pathway', and 'regulation of translation' gene ontologies (**Supplementary Figure**

5b). In sum, the DEGs between XX and XY PGCs were characterized by their sex-specific

roles in meiotic/mitotic cell cycle regulation, post-transcriptional processing of mRNA, and

cell-cell signaling properties (**Supplementary Figure 5a-b**). These analyses provide a

clear description of the transcriptional differences between XX and XY PGCs underlying the

transition from bipotential germ cells to sex-specific oogonium or gonocytes.

To quantitatively assess chromatin dynamics during PGC sex determination, we next

evaluated the number of differentially accessible chromatin peaks (DAPs) between XX and

XY germ cells (**Fig. 4b**). The DAP analyses ((log$_2$fold-change > 0.25) revealed 1,720, 2,311,

and 5,626 XX enriched DAPs and 3,341, 3,117, and 15,546 XY enriched DAPs at E11.5,

E12.5, and E13.5, respectively (**Fig. 4b).** The largest increase in the number of DA peaks

was found between XY PGCs (15,546) and XX PGCs (5,626) at E13.5. The DAPs for XX and

XY PGCs at E11.5-E13.5 were predominantly located in promoter, distal intergenic, or

intronic regions, indicating that these DAPs likely correspond to gene regulatory sites

(**Supplementary Figure 5c**). Together, these data suggest that the chromatin architecture

of PGCs, and especially the chromatin of XY PGCs, undergoes significant remodeling during

sex determination.

We next sought to associate sex-specific changes in chromatin accessibility with

changes in gene expression for PGCs. To determine peak-gene associations, we correlated

snATAC-seq peak accessibility with the expression of nearby genes, with the goal to

identify sex- and stage-specific chromatin peaks positively correlated with mRNA

expression (*p*-value < 0.05; z-score > 0). This analysis detected putative gene regulatory

interactions based on correlations between chromatin peak accessibility and gene

expression levels for all peaks and genes within 500 kb of each other[77]. Given the notable

increase in DA peaks between XX and XY PGCs at E13.5 (**Fig. 4b**), we first identified the

predicted target genes of all sex-specific DA peaks at this stage (**Supplementary Figure**

6a-b). For E13.5 XX PGCs, we observed positive DA peak-gene correlations for genes

enriched for biological functions such as 'WNT signaling pathway', 'chromosome

segregation', 'chromatin organization', 'meiotic cell cycle', and 'RNA stabilization'

(**Supplementary Figure 6a**). A similar analysis of E13.5 XY PGCs revealed positive DA

peak-gene correlations for genes involved in 'ncRNA metabolic process', 'mitotic cell cycle',

'DNA repair', 'regulation of translation', and 'RNA localization' (**Supplementary Figure**

6b). These findings demonstrate that we can use chromatin accessibility information to

identify the regulatory elements linked to sex-specific genes in PGCs.

We next investigated the associations of differentially accessible (DA) chromatin

peaks with differentially expressed (DE) gene expression for each PGC type. At E11.5, we

found that DA peak-to-gene linkages were associated with ~20% of DE genes in XX PGCs

and ~1% of DE genes in XY PGCs (**Supplementary Figure 6c**). The overlap of DA peak-to-

gene linkages with DE genes increased steadily at E12.5 and E13.5, with ~35% and ~60%

of DA peaks in E13.5 XX and XY PGCs, respectively, positively correlated with DE gene

expression (**Supplementary Figure 6c**). For example, at E13.5, we identified the DA peaks

positively correlated with the DE genes *Porcn* (involved in the processing of WNT

proteins)[71] and *Rec8* (involved in meiotic recombination)[8] in XX PGCs and *Rimbp1*

(regulates cytosolic calcium ion concentration)[78] in XY PGCs (**Fig. 4c-d; Supplementary**

Figure 6d). Immunofluorescence staining also confirmed the expression of *Porcn* in E13.5

fetal XX germ cells (**Supplementary Figure 7a**), demonstrating that single-nucleus

multiomics can identify DE genes and the DA chromatin peaks that are associated with

their expression.

A computational approach to identify candidate TFs regulating XX PGC gene

expression

To better utilize the multiomics data, we developed an analytical flow to identify

potential TFs that are involved in regulating the expression of DEGs in PGCs. First, we

classified peak status by identifying peaks that are differentially accessible between XX and

XY PGCs following the analytical framework in **Fig. 5a**. Second, we linked peaks to genes to

identify positive correlations between DA chromatin peaks and the expression of DEGs at

each embryonic stage (*p*-value < 0.05; z-score > 0; **Fig. 5a**). Third, we identified all enriched

TF binding motifs located within DA chromatin peaks and found unique motifs with a

significant FDR threshold (*p*-value < 0.05; **Fig. 5a**). We investigated only the TFs that were

differentially expressed between sexes and that showed germline enrichment. Finally, we

predicted the target genes of candidate TFs by determining the genomic position of their

enriched motifs and the genes linked to their resident peaks (**Fig. 5a**). This analytical flow

allowed us to rank enriched TF binding motifs based on variation in chromatin

accessibility, and unbiasedly infer TFs involved in PGC sex determination with greater

confidence.

In XX PGCs, we uncovered several candidate TF regulators of E11.5-E13.5 XX-

enriched DEGs (**Supplementary Figure 8a-c**). We plotted the mRNA expression levels of

TFs and the *in silico* chromatin binding score, termed 'motif activity', of all significantly

enriched binding motifs (**Supplementary Figure 8a-c**). For example, in E11.5 XX PGCs,

RREB1 is a differentially expressed TF showing high gene expression, but negative levels of

in silico chromatin binding (**Supplementary Figure 8a**). This finding indicates that

although RREB1 is a highly expressed TF in E11.5 XX PGCs, RREB1 does not likely promote

increased chromatin accessibility either because it does not bind to chromatin or a critical

co-factor is unavailable (**Supplementary Figure 8a**). Following this analytical framework,

we focused our analysis on TFs that showed high levels of gene expression, were

differentially expressed in XX PGCs, and displayed a positive *in silico* chromatin binding

score (**Supplementary Figure 8a-c**). The TFs meeting these criteria were KLF12, NR6A1,

and ZFX in E11.5 XX PGCs; TFAP2c in E12.5 XX PGCs; and BACH2, CREM, GATA2, GLI3,

MGA, NFKB1, PLAGL1, TBX4, TCFL5, TFAP2c, ZBTB7c, and ZFX in E13.5 XX PGCs

(**Supplementary Figure 8a-c**). Of these sixteen TF candidates with PGC-enriched

transcript expression, only seven (*Gata2, Mga, Nr6a1, Tbx4, Tcfl5, Tfap2c,* and *Zfx*) showed

enriched expression in germ cells when compared to the somatic compartment of the

gonad, and thus met our requirements for further *in silico* investigation (**Supplementary**

Figure 9a). These candidate TFs have reported roles in cell fate determination, growth and

fertility regulation, and meiotic progression[79,80], all of which are in line with the cellular

events that occur as PGCs commit to the female pathway. Nonetheless, the chromatin

binding and gene expression of GATA2, MGA, TBX4, TCFL5, and TFAP2c showed the

clearest patterns of sexual dimorphism in E13.5 PGCs and represented the most compelling

TF candidates (**Fig. 5b-c; Supplementary Figure 9b-f**).

The binding motif for TFAP2c (also known as *AP2γ*) showed prominent TF-

associated accessibility, especially when compared to all other significantly enriched TF

binding motifs (**Supplementary Figure 8b-c**). Immunofluorescence staining of TFAP2c

also showed enrichment of TFAP2c in E13.5 XX PGCs when compared to the somatic

compartment **Supplementary Figure 9g**). Compared to XY PGCs, XX PGCs had enriched

Tfap2c expression (p-value < 0.05; log2FC > 0.25) and enriched accessibility of the TFAP2c

motif at E12.5-E13.5 (**Fig. 5b**). The TFAP2c motif was close to genes related to glycogen

metabolism, carbohydrate metabolism, and chromatin modification (**Fig. 5d**). The

predicted target genes of TFAP2c accounted for 75% (372/494) of all DEGs with peak-to-

gene linkages in XX PGCs (**Fig. 5e**), suggesting that TFAP2c is likely a high-level regulator of

female PGC sex determination.

In addition to TFAP2c, we also investigated the regulatory potential of TCFL5

(Transcription Factor Like 5) in greater detail. TCFL5 was the most differentially expressed

in E13.5 XX PGCs (p-value < 0.05; log2FC > 0.25) (**Fig. 5c**). Accessibility of the TCFL5 motif

was also enriched in E13.5 XX PGCs, indicating that chromatin binding of TCFL5 is likely

strongest at this stage (**Fig. 5c**). The predicted target genes of TCFL5 totaled 74%

(367/494) of all DEGs with peak-to-gene linkages in XX PGCs (**Fig. 5e**). The TCFL5-

associated genes were related to chromosome segregation, meiotic cell cycle, sister

chromatid cohesion, histone modification, regulation of TF activity, and cell-cell signaling

by WNT. Our findings are supported by a recent report on the role of TCFL5 in XX PGCs,

which demonstrated the requirement of TCFL5 in meiotic progression and female

fertility[81].

Given that the motifs for TFAP2c and TCFL5 were not detected at every XX-enriched

DEG, we hypothesize that a network of TFs, such as those identified in **Supplementary**

Figure 9a, could work in cooperativity to regulate XX PGC gene expression. For example,

we found that the predicted target genes of ZFX, MGA, NR6A1, TBX4, and GATA2 accounted

for 73% (359/454), 31% (152/494), 20% (100/494), and 6% (32/494), respectively, of all

DEGs with peak-to-gene linkage in XX PGCs (**Fig. 5e**). We also found that TFAP2c, TCFL5,

ZFX, MGA, and NR6A1 may regulate the expression of each other (**Fig. 5f**). For instance, the

motif for ZFX was located in the enhancers or promoters for TFAP2c, TCFL5, MGA, and

NR6A1 (**Fig. 5f**). Likewise, NR6A1 is predicted to control the expression of TFAP2C, TCFL5,

and TBX4 (**Fig. 5f**). Furthermore, the motifs for the candidate TFs were statistically linked

to genes involved in 'meiotic cell cycle' and the 'WNT signaling pathway' (**Fig. 5g-h**).

Meiosis related genes such as *Tex15, Sycp1, Dmc1,* and *Hormad1* contained the motifs for at

least three of the predicted TFs in their regulatory sequences (**Fig. 5g**). A similar pattern

was also found for genes related to WNT signaling, e.g. *Pkd1, Notum, Porcn, Fzd3,* and *Eda*

were predicted to be regulated by the binding of at least two of the putative TFs in XX PGCs

(**Fig. 5h**). These data indicate that there may be unknown and undefined TF regulatory

networks underlying XX PGC identity and function.

Identification of candidate TF regulators of XY PGC differentiation

To identify candidate TF regulators involved in XY PGC sex determination, we

performed the same analysis as described for XX PGCs (**Fig. 5a**). Our analysis focused

strictly on differentially expressed TFs that show evidence of chromatin binding based on

the empirical *in silico* chromatin binding score (**Supplementary Fig. 10a-c**). In contrast to

XX PGCs (**Supplementary Fig. 8**), we did not identify any significantly enriched TF binding

motifs (p-value > 0.05) meeting our criteria in E11.5 XY PGCs (**Supplementary Fig. 10a**).

Only three XY-enriched TFs, FOXP1, POU6F2, and TCF4, were differentially expressed

between XY and XX PGCs at E12.5 (**Supplementary Fig. 10b**), and only TCF4 showed a

positive *in silico* chromatin binding score in E12.5 XY PGCs (**Supplementary Fig. 10b**). For

E13.5 XY PGCs, we detected five significantly enriched motifs that bound differentially

expressed TFs (p-value < 0.05): FOXK2, FOXO1, FOXP1, POU6F1, and TCF4

(**Supplementary Fig. 9c**). Of these six XY-enriched TF candidates from E11.5 to E13.5,

FOXO1, FOXK2, and POU6F1/2 emerged as the top candidates based on germline enriched

expression when compared to somatic cells, differential expression in XY PGCs, and

positive *in silico* chromatin binding scores (**Supplementary Fig. 11a-b; Fig. 5i-j**).

Together, these data indicate that XY PGCs rely less on differentially expressed TF-

mediated gene regulation when compared to XX PGCs.

At E13.5, when the most TFs and their motifs were found in XY PGCs, we observed

significant enrichment of the FOX (forkhead-box) protein family of TFs (**Supplementary**

Fig. 10c). FOX proteins contain pioneering transcription properties that allow for binding

to condensed chromatin and enabling competency of gene activity through histone

remodeling[82]. Given their role in opening condensed chromatin, FOX TFs play important

roles in regulating the expression of genes involved in cellular differentiation, proliferation,

plasticity, and growth[49]. In particular, we found that the expression of *Foxo1* and *Foxk2*

were specific to XY PGCs when compared to XX germ cells and the XY somatic compartment

(**Supplementary Fig. 10c; Supplementary Fig. 11a; Fig. 5i**). From our dataset, we found

the FOXK2 motif positively associated with genes related to cellular proliferation, cell cycle

arrest, mitosis, and DNA damage checkpoint (**Fig. 5k**). In addition, the predicted target

genes of FOXK2 totaled 26% (54/208) of all DEGs with peak-to-gene linkages in XY PGCs

(**Fig. 5l**). This finding was consistent with the number of predicted target genes of FOXO1,

which included 28% (58/208) of all DEGs with peak-to-gene linkages in XY PGCs and may

be the result of motif redundancy among FOX proteins. Given the substantial increase in

the number of DA peaks in E13.5 XY PGCs (**Fig. 4b**), we hypothesize that the pioneering

properties of the FOX family promotes chromatin remodeling as XY PGCs transition to

gonocytes.

We also detected significant enrichment of the POU domain, class 6 (POU6) TF

family in XY PGCs (**Supplementary Fig. 10b-c; Supplementary Fig. 11a & c; Fig. 5j**). The

POU family plays important roles in regulating cellular differentiation and the timing of

cellular events[83]. We found XY-enriched expression of POU6F1 and POU6F2 in PGCs,

however, only POU6F2 displayed germline enrichment when compared to the somatic cell

populations of the gonad (**Supplementary Fig. 11a & c; Fig. 5j**). Furthermore, we

observed the strongest POU6F1 and POU6F2 *in silico* chromatin binding scores in XY PGCs

at E13.5 (**Supplementary Fig. 11c; Fig. 5j**). The POU6F1/2 motif was associated with

19% (40/208) of all DEGs with peak-to-gene linkages in XY PGCs (**Fig. 5l**). POU6F1/2-

associated genes were related to voltage-gated chloride channel activity (*Ano6*)[84],

regulation of cell proliferation (*Appl1*)[85], regulation of actin filaments (*Fmnl2*)[86], Notch

signaling (*Maml3*)[87], lipid metabolism (*Osbpl9*)[88], and ubiquitin ligase activity (*Usp7*)[89] (**Fig.**

5m). Thus, the POU6 TF family is a top candidate to coordinate gene expression activity in

XY PGCs.

We next compared the number and identity of FOXO1-, FOXK2-, and POU6F1/2-

associated genes in XY PGCs. First, we did not detect the FOXO1, FOXK2, or POU6F1/2

motifs in the promoters/enhancers for each of these TFs. These findings indicate that these

TFs do not regulate the expression of each other, and other mechanisms or regulatory

pathways are required to activate the expression of candidate TFs in XY PGCs.

Furthermore, the predicted target genes of the FOX and POU6 TF families shared little

overlap with each other (11/87) (**Fig. 5m**). The genes that were shared between FOXO1,

FOXK2, and POU6F1/2 had reported functions in maintaining survival after DNA damage

(*Alkbh8*)[90], TGFβ signaling (*Bmp7*)[91], and initiation of mitosis (*Nek7*)[92] (**Fig. 5m**). By

comparison, the predicted target genes of FOXO1 and FOXK2 showed considerable overlap

(42/47) and included genes related to RNA splicing (*Aqr*)[93], cell cycle progression (*Cdk6*)[94],

cellular metabolism (*Eno1* and *Odc1*)[95,96], cell death (*Pml*)[97], and negative regulation of the

IGF1 signaling pathway (*Socs2*)[98] (**Fig. 5m**). The motifs for FOXO1, FOXK2, and POU6F1/2

were statistically associated with genes involved in 'mitotic cell cycle' and 'signal

transduction' (**Fig. 5n-o**). Mitosis-related genes such as *Cradd, Cdk6, Exoc6b,* and *Pml* were

potentially regulated by FOXO1 and FOXK2 (**Fig. 5n**). In addition, we noted that the

POU6F1/2 motif was associated with *Cradd* and *Psmg2* (**Fig. 5n**). We observed a similar

pattern for genes related to signal transduction (**Fig. 5o**). For example, *Bmp7, Map3k5,*

Akt3, and *Sulf1* were associated with FOXO1 and FOXK2, whereas *Bmp7, Map3k5,* and

Sfprp1 were associated with POU6F1/2 motif activity (**Fig. 5o**). Taken together, these data

suggest that in XY PGCs, although FOXO1, FOXK2, and POU6F1/2 regulate genes with

similar properties, the FOX and POU6 TF families are unlikely to collaboratively regulate

gene expression.

Sex-specific enrichment of ligand-receptor signals between gonadal support cells

and PGCs

Sexual dimorphism of germ cell differentiation is thought to be controlled by a

meiosis-inducing substance produced by the somatic cells of the fetal ovary and/or by a

meiosis-preventing substance produced by the fetal testis[99]. In experimentally generated

XX/XY chimeras, both XX and XY PGCs can initiate meiosis when residing in the fetal ovary

or enter mitotic arrest when colonizing the fetal testis[100]. In the fetal ovary, PGC meiosis is

induced by retinoic acid (RA) produced by ovarian and mesonephric somatic cells[8].

Consistent with this model, RA signaling induces expression of *Stra8* and *Meiosin*, both of

which are critical for promoting entry into meiosis[8]. Signaling by WNT and BMP also

influences the timing of meiotic onset in XX PGCs[8], and inhibin/activin signaling regulates

XX germ cell growth and proliferation[101]. In the fetal testis, RA degradation by CYP26B1

and FGF9 signaling from XY supporting cells result in reduced *Stra8* expression,

consequently blocking meiotic entry in XY PGCs[8]. Thus, PGC sex determination is

dependent on external signals from the gonadal environment rather than chromosomal

sex.

To uncover potential instructive signals from the supporting somatic cells (Sertoli

cells in the fetal testis or granulosa cells in the fetal ovary) to PGCs, we searched for

supporting cell-derived ligands and the corresponding receptors in PGCs (**Fig. 6a**), using

CellphoneDBv5.0 for cell communication analysis[45]. We first identified the supporting cell

clusters from the snRNA-seq dataset of whole fetal gonads using the established female and

male supporting cell markers *Runx1* and *Sox9*, respectively[30,102]. We next quantified the

number of significant interactions between supporting cells and PGCs at each embryonic

stage (*p*-value < 0.05; **Fig. 6b**). Such analyses revealed that the number of interactions

gradually increased from E11.5 to E13.5 for both XX and XY gonads (**Fig. 6b**). We also

observed that the number of ligand-receptor pairs was 15-38% higher in XX gonads,

compared to XY gonads at the same embryonic stage (**Fig. 6b**). To validate our

computational approach, we next searched for known pathways associated with PGC

differentiation, such as signaling by WNT, inhibin/activin, and BMP (**Fig. 6c**). The number

of significant interactions per pathway increased in both XX and XY gonads from E11.5 to

E13.5 (**Fig. 6c; Supplementary Fig. 12a-b**). We also observed a moderate increase in the

number of interactions for the inhibin/activin and BMP pathways in XX gonads when

compared to XY gonads at the same embryonic stage (**Fig. 6c**). Finally, we found that the

number of interactions related to WNT signaling were nearly 2-3 times greater in XX than

XY gonads (**Fig. 6c; Supplementary Fig. 11a-b**). Together, these data demonstrate that

sexually dimorphic patterns of cell signaling events were present between supporting cells

and PGCs.

We next searched for new sex-specific enrichment of ligand-receptor pairs between

supporting cells and PGCs (**Fig. 6d-i**). For each embryonic stage, we first identified which

signaling pathways were detected in XX alone, XY alone, or both XX and XY gonads that met

our statistical threshold (p-value < 0.05; **Fig. 6d, f, & h**). At E11.5, XX and XY gonads shared

the greatest overlap of supporting cell-derived ligands and PGC-expressing receptors (**Fig.**

6d). The shared pathways between XX and XY gonads were largely related to adhesion by

collagen/integrin, fibronectin, and cadherin. E11.5 XX gonads had enrichment of 19 ligand-

receptor pairs related to, for example, BMP, ephrin, integrin, FGF, and WNT signaling (**Fig.**

6d). By contrast, E11.5 XY gonads had significant enrichment of 10 ligand-receptor pairs

predominantly related to cell adhesion pathways like ephrin, nectin, and integrin (**Fig. 6d**).

To highlight the general trends of expression between sexes, we compared the mean

expression of all representative ligand-receptor pairs from each group in Fig. 6d, f, & h (**Fig.**

6e, g, & i). In E11.5 gonads, we observed dimorphic expression of XX-enriched interactors,

such as BMP2/BMR1A/AVR2B and WNT4/FZD10/LRP5, but not the XY-enriched

interactors CADM1/NECTIN3 and EFNB1/EPHB3 (**Fig. 6e**). Furthermore, we did not

observe a sex bias for the mean expression of interacting pairs for the shared pathway

DLK1:NOTCH3 between E11.5 XX and XY gonads (**Fig. 6e**). These data indicate that at

E11.5, sexual dimorphism of signaling pathways, in terms of number and mean expression,

is most prominent between XX supporting cells and XX PGCs.

In E12.5 and E13.5 gonads, we observed a stronger XX bias in ligand-receptor

interactions between supporting cells and PGCs (**Fig. 6f-i**). At E12.5, we observed 49 and

24 significant interactions in XX and XY gonads, respectively (**Fig. 6f**). In E12.5 XX gonads,

BMP, Insulin-like growth factor (IGF), R-spondin, and WNT signaling were the most

frequently observed pathways and displayed strong XX-enriched mean expression (**Fig. 6f-**

g). In XY gonads, detection of new signaling pathways emerged from E11.5 to E12.5,

including signaling by BMP6, chemokines, Matrix metalloproteinases (MMP), and

Neuregulin (**Fig. 6f**). Compared to E12.5, E13.5 XX gonads continued to have enrichment

for the BMP, IGF, and WNT signaling pathways with additional pathways related to midkine

(MDK), Calsyntenin (Clstn), and SLIT and NTRK-like protein signaling detected (**Fig. 6f and**

h). The BMP5:BMR1A:AVR2B and MDK:PTPRZ1 ligand-receptor interactors also showed

XX-biased mean expression (**Fig. 6i**). Similarly in E12.5 and E13.5 XY gonads, we observed

maintenance of the ligand-receptor pairs, BMP6:ACVR:1A2B and BMP6:BMR1A:AVR2B

(**Fig. 6f and h; Supplemental Data 1**). However, enrichment of signaling by Desert

hedgehog, Notch, and WNT inhibition became significant in E13.5 XY gonads but not E12.5

(**Fig. 6f and h**). High mean expression of DHH:PTCH:GAS1 and DLK1:NOTCH3 interactors

was detected in E13.5 XY gonads but became indetectable in E13.5 XX gonads (**Fig. 6i**).

Taken together, these data suggest that sex-specific enrichment of ligand-receptor

interactions become gradually more apparent from E11.5 to E13.5.

Discussion

Male and female germ cells carry the hereditary information required for the generation of a new individual and the perpetuation of sexually reproducing species. Errors in germ cell development and differentiation can result in a variety of reproductive diseases, such as infertility[6]. Disruption of gametogenesis during early embryonic development can even lead to the onset of disease later in life, a concept which is supported by the Developmental Origins of Health and Disease (DOHaD) theory[103]. Thus, it is of critical importance to understand the molecular and epigenetic signatures of developing germ cells, and particularly PGCs. Nonetheless, the heterogeneous nature and low cell numbers of PGC populations often hinders the identification of novel factors that influence PGC fate. To overcome this limitation, we generated the first single-nucleus atlas of gene expression and chromatin accessibility for murine PGCs during sex determination. We demonstrate that single-nucleus multiomics successfully captures PGC sex determination at high resolution, identifies putative TFs involved in PGC sex determination, and provides insight into the cellular communication pathways between PGCs and the supporting cells of the gonads.

Integrated RNA and ATAC data for individual PGCs identified significant heterogeneity in XX PGC populations compared to XY PGCs.

We first identified the sub-groupings of E11.5-E13.5 XX and XY PGCs and resolved the patterns of gene expression and chromatin accessibility underlying each PGC population. We observed that the combined analysis of integrated snRNA- and snATAC-seq data produced better clustering and separation of PGCs than either assay alone. For example, we found that at E11.5, XX and XY PGCs share highly similar transcriptomic and

open chromatin profiles, indicating that PGCs at this stage are bipotential at the level of

gene regulation and chromatin accessibility. This observation agrees with the study that

showed the global overlap of transcriptomes between E11.5 PGCs from both sexes, in

which they detected only 101 DEGs between XX and XY PGCs with functional annotations

related to chaperonin complexes and proteasome formation[104]. At E12.5, subpopulations of

PGCs clustered separately within each sex, supporting the model that PGCs do not commit

to a sex-specific fate in a synchronous manner[54]. At E13.5, two sub-clusters of XX PGCs

appear, consistent with the asynchronous entry of XX PGCs into meiosis[54]. Others also

reported substantial heterogeneity of human and mouse PGC populations, often arising

from   intrinsic   variation   in   the   epigenetic   reprogramming   required   for   sex

differentiation[105,106].   These data underscore the discovery potential of simultaneously

measuring multiple layers of gene regulation to define the developmental fate of PGCs.

Molecular signatures of differentiating PGCs revealed disease-related genes

associated with PGC clusters.

When   examining   genes   that   could   be   responsible   for   the   expression-based

clustering of PGCs, we found TFs, cell cycle regulators, chromatin modifiers, and ribosome

biogenesis genes underlying the diverse transcriptional profiles of differentiating PGCs.

Several of the expressed genes enriched in individual PGC clusters were involved in

infertility or disease processes when nonfunctional. For example, mutations in *Lars2*, the

mitochondrial leucyl-tRNA synthetase, is associated with premature ovarian failure in

women[50] and aberrant methylation at the promoter of *Mgmt,* a gene that is required for

DNA repair and linked to the formation of testicular germ cell tumors in humans[51]. The

dynamic and varied regulatory profiles of PGCs that we observed highlight the necessity of

pairing chromatin accessibility information with gene expression data for elucidating the

mechanisms underlying PGC sex determination.

Sexual dimorphism in chromatin accessibility increases temporally during PGC

development.

The chromatin landscape of PGCs is the most sexually dimorphic at E13.5 when

compared to E11.5 and E12.5. The substantial increase in DAPs at E13.5 leads to several

hypotheses that could account for the changes to PGC chromatin architecture during sex

determination. First, it is possible that the chromatin of bipotential E11.5 PGCs is primed

for activating either sex-specific differentiation pathway. Chromatin priming occurs when

chromatin is maintained in an accessible state prior to gene expression, and is evidenced in

a number of developmental contexts, such as during specification of hematopoietic cells

and lineage commitment of embryonic stem cells[107,108]. During development, chromatin

priming fine-tunes gene activation by enabling a rapid response to external cues that

induce cell differentiation[107]. Second, we posit that E11.5 PGCs have fewer DA peaks

because they are actively dividing. Mitotic chromosome condensation is known to induce a

state of heterochromatin and the eviction of TFs from chromatin in actively dividing

cells[109]. Third, the increased number of DA peaks at E13.5 may be the result of changes to

chromatin structure as XX PGCs enter meiotic prophase I, an event that requires dramatic

changes to meiotic chromosomes as they undergo DNA double-strand breaks, synapsis, and

crossing over[47]. Indeed, increased chromatin accessibility was found in mouse

spermatocytes during prophase I of meiosis[110]. Finally, the upregulated expression of

forkhead TFs in male PGCs at E12.5 and E13.5 may induce the *de novo* establishment of DA

peaks through their pioneering activity. Nonetheless, our results are consistent with

previous reports that demonstrate the global decrease in DNA methylation levels, or

increased chromatin accessibility, in mouse PGCs beginning at E9.5 and continuing through

E13.5[111].

Single-nucleus multiomics identifies evidence for chromatin priming during PGC

lineage commitment.

Moreover, not all sex- and stage-specific DA chromatin peaks account for changes in

differential gene expression for PGCs. Chromatin accessibility and gene expression are

often asynchronously correlated during cellular differentiation, such as during chromatin

priming[77]. For example, accessibility of regulatory loci involved in cell-fate decisions may

precede gene expression to prime cells towards one developmental lineage versus

another[77]. In the context of germ cell differentiation, PGCs must also undergo extensive

chromatin remodeling events, including genome-wide DNA demethylation and a reduction

in both repressive and activating histone modifications, prior to sex determination[112].

Indeed, chromatin priming has been observed in PGCs from other species, such as

macaques, pigs, and goats[105]. Thus, it is likely that the DA chromatin peaks observed for

E11.5 and E12.5 PGCs in mice may either reflect global chromatin remodeling events

associated with sex determination or foreshadow future cell fate decisions by remaining

poised for TF binding. Nevertheless, a comprehensive profile of 3D chromatin structure

and enhancer-promoter contacts in differentiating PGCs is needed to fully understand how

changes to chromatin facilitate PGC sex determination.

The gene regulatory networks of XX PGCs are enriched for the transcription factors,

TFAP2c, TCFL5, GATA2, MGA, NR6A1, TBX4, and ZFX

Our multiomics approach uncovered compelling TF candidates that may be involved

in PGC development and differentiation. In XX PGCs, seven TF prospects were identified:

GATA2, MGA, NR6A1, TBX4, ZFX, TCFL5, and TFAP2c. Of these TFs, TCFL5 and TFAP2C

showed the strongest enriched expression in XX PGCs. TCFL5 binds to the promoters of

meiotic genes such as *Sycp1* (synapsis) and *Stag3* (cohesion) during spermatogenesis in the

mouse[79]. *Tcfl5* is indispensable for the completion of meiosis and spermatid maturation[79],

and meiosis of female germ cells[81]. Our multiomics data also demonstrate strong *Tfap2c*

expression and motif enrichment in XX PGCs beginning at E12.5 and continuing through

E13.5. These findings are corroborated by other single-cell RNA-seq analyses that showed

Tfap2c expression in human and mouse PGCs and gonocytes[80,113-116]. Additionally, TFAP2c

has several reported roles in cell-type specification in both mouse and human, including

germline specification and maintenance of germ cell identity[80], remodeling of naïve

enhancers[117], and hormone responsiveness[118]. Loss of TFAP2c in mice also leads to a

reduction in germ cell number by either PGC apoptosis or PGC differentiation into somatic

cells[119]. Considering that changes in gene regulation leading to the female and male

pathways begin as early as E12.5[3], understanding how TCFL5 and TFAP2c activity is

induced and investigating the role of TFAP2c in PGC sex determination are important

future directions.

XY PGCs showed sex-specific enrichment of transcription factors associated with reproductive dysfunction.

In XY PGCs, we detected significant enrichment of the forkhead box and POU6 families of TFs. In particular, FOXO1, FOXK2, and POU6F1/2 exhibited XY biased expression and *in silico* chromatin binding. Although the function of *Foxo1* has been studied in gonocytes[120], little is known about the roles of *Foxk2* and *Pou6f1/2* in PGCs. Loss of *Foxo1* in embryonic gonocytes leads to defective spermatogenesis in adulthood[120], and inactivation of *Foxo1* in spermatogonia results in germ cell loss and male infertility[121]. FOXO1 activity is also widely involved in regulating metabolic homeostasis, redox balance, cell death, and DNA damage repair[122]. Likewise, FOXK2 functions in similar roles as FOXO1, such as in cellular metabolism, cell survival, and apoptosis[123]. Furthermore, the POU6 family consists of POU6F1 and POU6F2, which share highly conserved DNA motif recognition sequences and function in similar cellular pathways[124]. Both POU6F1 and POU6F2 have been reported to suppress tumor proliferation[125], and POU6F2 mutations have been implicated in the progression of Wilms Tumor, hypogonadism, and pubertal failure in human patients[126]. Investigating the cooperativity of these TFs will greatly improve our understanding of how mitotic arrest and the commitment to spermatogenesis are regulated during germ cell development.

Single-nucleus RNA-seq maps the temporal expression patterns of WNT, BMP, and RA signaling during PGC sex determination.

During sex determination, PGCs respond to signals from the supporting cells of the gonads to determine their sex-specific fate. In the fetal ovary, *Runx1+* supporting cells

produce WNT4 to maintain germ cell pluripotency and prevent precocious meiotic

entry[8,60]. We observed signaling by WNT4, WNT5A/B, and WNT6 in both XX and XY gonads

as early as E11.5. The number of interactions related to WNT signaling also increased from

E11.5 to E13.5 in both XX and XY gonads even though diminished WNT signaling is

required for meiotic entry[60]. However, XY PGCs showed enrichment of the WNT agonist

SFRP2[127] at E13.5, indicating that WNT signaling may be inhibited in XY PGCs earlier than

XX PGCs. BMP secretion by ovarian supporting cells also plays a critical role in regulating

meiotic entry[8]. Even though BMP6 signaling was observed in XY PGCs, BMP2 and BMP5

signaling were specific to XX PGCs beginning at E11.5. Our findings are consistent with

functional studies showing that BMP2 signaling induces *Zglp1* expression and promotes the

oogenic fate[10]. Additionally, RA produced by both ovarian and mesonephric somatic cells is

required to initiate expression of *Stra8* and *Meiosin*, two TFs that are essential for

completion of meiosis[8,9]. Significant enrichment of the RA signaling pathway was not

detected between supporting cells and PGCs in XX or XY gonads at any embryonic stage

with CellphoneDB. Given that RA is primarily produced by the mesonephros[66], detection of

RA signaling between gonadal supporting cells and PGCs is unlikely. However, *in silico*

chromatin binding of RA receptors was enriched in XX PGCs and the RA receptor motifs

were found in the regulatory loci for *Rec8, Stra8, Sycp2, Sycp3,* and *Zglp1*, which is

consistent with previous studies[128]. Taken together, our findings provide insight into the

temporal patterns of WNT, BMP, and RA signaling during PGC differentiation.

Discovery analysis identifies potentially new signaling pathways between gonadal

supporting cells and PGCs.

In addition to these pathways known for their roles in PGC sex determination, we

identified several uncharacterized ligand-receptor pairs with sex-specific enrichment. For

example, midkine (MDK) signaling was enriched in E13.5 XX gonads. Although the role of

MDK signaling in PGCs is not understood, MDK is known to be important for maintaining

cell survival and growth and promoting cell migration[129]. MDK is a heparin-binding

cytokine and, together with pleiotrophin, regulates female reproductive activities such as

oocyte maturation and the estrous cycle[130]. Furthermore, in E13.5 XY gonads, we found

enrichment of the Desert Hedgehog (DHH) signaling pathway between *Sox9+* cells and XY

PGCs. In addition to regulating fetal Leydig cell differentiation[131], DHH signaling has also

been shown to regulate germ cell maturation and spermatogenesis[132]. Ablation of *Dhh* in

adult XY mice causes infertility due to a loss of mature sperm[132], however, whether *Dhh*

regulates germ cell development in fetal XY mice remains to be determined. Overall, we

posit that these and other signaling pathways are important for promoting PGC survival

and differentiation.

Our study demonstrated the benefit of using multiomics approaches to probe the

cellular and developmental pathways involved in cell fate decisions, gametogenesis, and

PGC differentiation. Unlike traditional assays, joint RNA and ATAC analyses from individual

PGCs enabled the detection of paired chromatin accessibility and gene expression

information from the same cell to discover new gene regulatory interactions. We show that

our multiomics data accurately identified enrichment of known pathways involved in PGC

sex determination, suggesting that our computational approaches are valid and capable of

discovering potentially new regulators of PGC fate. Indeed, our findings revealed the

diversity of factors required for PGC programming. For example, we cataloged the

heterogeneity of PGC populations based on chromatin status and transcription, identified

chromatin priming as a major regulatory mechanism in PGC lineage commitment, and

revealed a core set of TFs and ligand-receptor pairs unique to XX and XY PGCs. Taken

together, our data demonstrate the power of explorative single-nucleus ATAC-seq and

RNA-seq to understand the mechanisms inherent to germline differentiation.

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1025 **Conflict of interest Statement (if requested by the journal)**

1026 The authors declare no competing financial or non-financial interests.

 Figure legends

 Figure 1: Single-nucleus multiomics sequencing of E11.5-E13.5 XX and XY PGCs. (a)

 Workflow of single-nucleus multiomics analyses. (b-c) Dimensional reduction (UMAP) and

 cell-type specific clustering of snRNA-seq data collected from E11.5-E13.5 XX and XY PGCs.

 Each dot represents an individual cell color coded by either embryonic stage and sex (a) or

 cluster number (c). (d) Cell type composition of snRNA-seq clusters. The embryonic stage

 and sex of the PGC population is indicated on the *x*-axis and the unbiased clustering

 number is represented on the *y*-axis. (e-f) UMAP of snATAC-seq data collected from E11.5-

 E13.5 XX and XY PGCs color coded by either embryonic stage and sex (e) or cluster number

 (f). (g) Cell type composition of snATAC-seq clusters. (h-i) UMAP visualization of E11.5-

 E13.5 XX and XY PGCs using a joint, or weighted, measurement of snRNA- and snATAC-seq

 modalities. (h) Trajectory of transcriptomic changes (also known as pseudo-time analysis

 in Monocle) among PGCs overlaid on the joint UMAP. Arrows indicate the path of the

 developmental trajectory. Numbers on the pseudotime trajectory indicate the following: '0'

 represents the starting cell population; '1' indicates the branching point in the trajectory

 path; and '2' represents the terminal cell populations of the trajectory paths. Each PGC is

 color coded by embryonic stage and sex. (i) Unbiased clustering of PGCs using combined

 snRNA- and snATAC-seq data. Each PGC is color coded by cluster number. (j) Cell type

 composition of joint UMAP clusters.

 Figure 2: Molecular characterization of genes known for their roles in male PGC

 differentiation. (a) Dotplot of the average expression and percentage of cells expressing

 Rb1, Rbl2, Cdkn1b, Cdkn2b, Bnc2, Cnot1, Dnd1, Nanos2, Nanos3, and *Zkscan5.* The DNA

binding motif for ZKSCAN5 is indicated above. The color scale represents the average expression level, and the size of the dot represents the percentage of cells expressing the gene. (b) *Left:* Coverage plot of the normalized snATAC-seq signal at the *Bnc2* locus. *Right:* Violin plot of *Bnc2* expression in E11.5-E13.5 XX and XY PGCs. (c) Joint UMAP showing *Bnc2* expression levels for E11.5-E13.5 XX and XY PGCs. Individual cells are color coded by the level of *Bnc2* expression. (d) Candidate factors (*x*-axis) regulating *Bnc2* expression based on their Cistrome DB regulatory potential score (*y*-axis). (e) Dotplot of the average expression and percentage of cells expressing the candidate factors that potentially regulate *Bnc2* expression. The color scale represents the average expression level, and the size of the dot represents the percentage of cells.

Figure 3: Molecular characterization of genes known for their roles in female PGC sex determination. (a) Dotplot of the average expression and percentage of cells expressing *Rec8, Sycp2, Sycp3, Ctnnb1, Meioc, Rnf2, Stra8, Ythdc2, Zglp1*, and *Dmrt1*. The DNA binding motif for DMRT1 is indicated. The color scale represents the average expression level, and the size of the dot represents the percentage of cells expressing the gene. (b) *Left:* Coverage plot of the normalized snATAC-seq signal at the *Stra8* locus. *Right:* Violin plot of *Stra8* expression in E11.5-E13.5 XX and XY PGCs. (c) Joint UMAP showing *Stra8* expression levels for E11.5-E13.5 XX and XY PGCs. Individual cells are color coded by the level of *Stra8* expression. (d) Candidate factors (*x*-axis) regulating *Stra8* expression based on their Cistrome DB regulatory potential score (*y*-axis). (e) Dotplot of the average expression and percentage of cells expressing the candidate factors that potentially regulate *Stra8*

1072　　expression. The color scale represents the average expression level, and the size of the dot

1073　　represents the percentage of cells.

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Figure 4: Identification of differentially expressed genes and differentially accessible

peaks in E11.5-E13.5 XX and XY PGCs. (a) Bar graph showing the number of differentially

expressed genes (DEGs) between XX and XY PGCs at E11.5-E13.5. (b) Bar graph showing

the number of differentially accessible chromatin peaks (DAPs) between XX and XY PGCs at

E11.5-E13.5. (c) *Left:* Coverage plot of the normalized snATAC-seq signal at the DEG *Porcn*

locus. Peak-to-gene linkages are indicated by the 'links' line. *Right:* Violin plot of *Porcn*

expression in E11.5-E13.5 XX and XY PGCs. (d) *Left:* Coverage plot of the normalized

snATAC-seq signal at the DEG *Rimbp1* locus. Peak-to-gene linkages are indicated by the

'links' line. *Right:* Violin plot of *Rimbp1* expression in E11.5-E13.5 XX and XY PGCs.

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Figure 5: Analysis of gene networks in E11.5-E13.5 XX and XY PGCs. (a) Illustration of

the analytical flow to infer gene networks (*i.e.* transcription factors (TFs) and their

predicted target genes) involved in PGC sex determination. (b-c; i-j) Line plots showing the

expression levels and *in silico* chromatin binding, termed 'motif activity', of *Tfap2c* (b)*, Tcfl5*

(c)*, Foxk2* (i)*,* and *Pou6f2* (j) in E11.5-E13.5 XX (pink) and XY (teal) PGCs. The *p*-value of

motif enrichment and the TF binding motif are indicated. (d and k) Enrichment analysis of

biological process gene ontology (GO) of the predicted target genes for TFAP2c (d) and

FOXK2 (k). The color scale represents the *p*-value of GO term enrichment, and the size of

the dot indicates the gene ratio for each GO term. (e and l) The number of predicted target

genes for enriched TFs in XX PGCs (e) and XY PGCs (l). (f) Circos plot showing whether the

motifs for TFAP2C, TCFL5, ZFX, MGA, or NR6A1 (indicated in black) are detected in the

regulatory loci linked to *Tbx4, Nr6a1, Mga, Tcfl5,* or *Tfap2c* (indicated in grey). (m) Venn

diagram showing the overlap of predicted target genes for POU6F1/2, FOXK2, and FOXO1.

(g-h) Icon array showing the presence (pink/purple) or absence (light grey) of the motifs

for TFAP2c, TCFL5, ZFX, MGA, NR6A1, TBX4, and GATA2 in peaks linked to genes related to

meiosis (g) or WNT signaling (h). (n-o) Icon array showing the presence (teal) or absence

(light grey) of the motifs for FOXO1, FOXK2, and POU6F1/2 in peaks linked to genes related

to mitosis (n) or signal transduction (o).

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Figure 6: Sexually dimorphic interactions between PGCs and gonadal supporting

cells. (a) Illustration of cell communication between PGCs and gonadal supporting cells. (b)

Number of significant interactions or ligand-receptor pairs (*p*-value < 0.05) between

supporting cells and PGCs in E11.5-E13.5 XX and XY gonads. (c) Number of significantly

enriched ligand-receptor pairs, or interactions, per signaling pathways induced by either

WNTs, inhibins/activins, or BMPs in E13.5 XX and XY gonads. (d, f, & h) Venn diagrams

showing the overlap of significant interactions between supporting cells and PGCs in XX

and XY gonads at E11.5 (d), E12.5 (f), and E13.5 (h). (e, g, & i) Dotplots showing the mean

expression for all interacting partners in representative ligand-receptor pairs in XX and XY

gonads at E11.5 (e), E12.5 (g), and E13.5 (i). Ligand-receptor pairs in pink represent XX-

specific interactions, ligand-receptor pairs in blue represent XY-specific interactions, and

ligand-receptor pairs in grey represent interactions found in both XX and XY gonads. The

size of the dot represents the mean expression value.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

a Supporting Cell / PGC

b Significant Interactions (p < 0.05) Supporting Cell to PGC
Number of Interactions vs E11.5 XX, E11.5 XY, E12.5 XX, E12.5 XY, E13.5 XX, E13.5 XY

c Number of Interactions per Pathway
Signaling by WNT, Signaling by Inhibin/Activin, Signaling by BMP
# of Sig. Interactions (p < 0.05)
E13.5 XX, E13.5 XY

Ligand-Receptor Pairs at E11.5

d E11.5 XX (19), 47, E11.5 XY (10)
BMP2_BMR1A_AVR2B
EFNA2_EPHA4
FN1_integrin_aVb1_complex
NCAM1_FGFR1
PPIA_BSG
WNT4_FZD10_LRP5
CADM1_NECTIN3
EFNB1_EPHB3
NECTIN1_NECTIN3
SPP1_integrin_aVb3_complex

e Mean Expression Values for All Interacting Partners
E11.5 XX Runx1+ to PGC
E11.5 XY Sox9+ to PGC
Mean Value 0.50, 1.00, 1.50, 1.75
BMP2 BMR1A AVR2B / WNT4 FZD10 LRP5 / DLK1 NOTCH1 / CADM1 NECTIN3 / EFNB1 EPHB3

Ligand-Receptor Pairs at E12.5

f E12.5 XX (49), 42, E12.5 XY (24)
BMP2_BMR1A_AVR2B
BMP5_ACVR1_BMPR2
IGF2_IGF2R
RSPO1_Kremen1_LRP6
WNT5A_ROR2
BMP6_ACVR_1A2B_receptor
CX3CL1_CX3CR1
MMP2_integrin_aVb3_complex
NRG1_ERBB3
NTN4_NTRK2

g Mean Expression Values for All Interacting Partners
E12.5 XX Runx1+ to PGC
E12.5 XY Sox9+ to PGC
Mean Value 0.50, 1.00, 1.50, 1.75
BMP5 ACVR1 BMPR2 / WNT5A ROR2 / COL4A1 ADGRG6 / BMP6 ACVR 1A2BRr / MMP2 integrin _aVb3 complex

Ligand-Receptor Pairs at E13.5

h E13.5 XX (57), 43, E13.5 XY (30)
BMP5_BMR1A_AVR2B
MDK_PTPRZ1
CLSTN1_NRXN1
SLIT2_ROBO1
WNT4_FZD3_LRP5
BMP6_BMR1A_AVR2B
DHH_PTCH_GAS1
DLK1_NOTCH3
WNT5B_SFRP2
WNT6_SFRP2

i Mean Expression Values for All Interacting Partners
E13.5 XX Runx1+ to PGC
E13.5 XY Sox9+ to PGC
Mean Value 0.50, 1.00, 1.50, 1.75
BMP5 BMR1A AVR2B / MDK PTPRZ1 / NECTIN1 NECTIN3 / DHH PTCH GAS1 / DLK1 NOTCH3