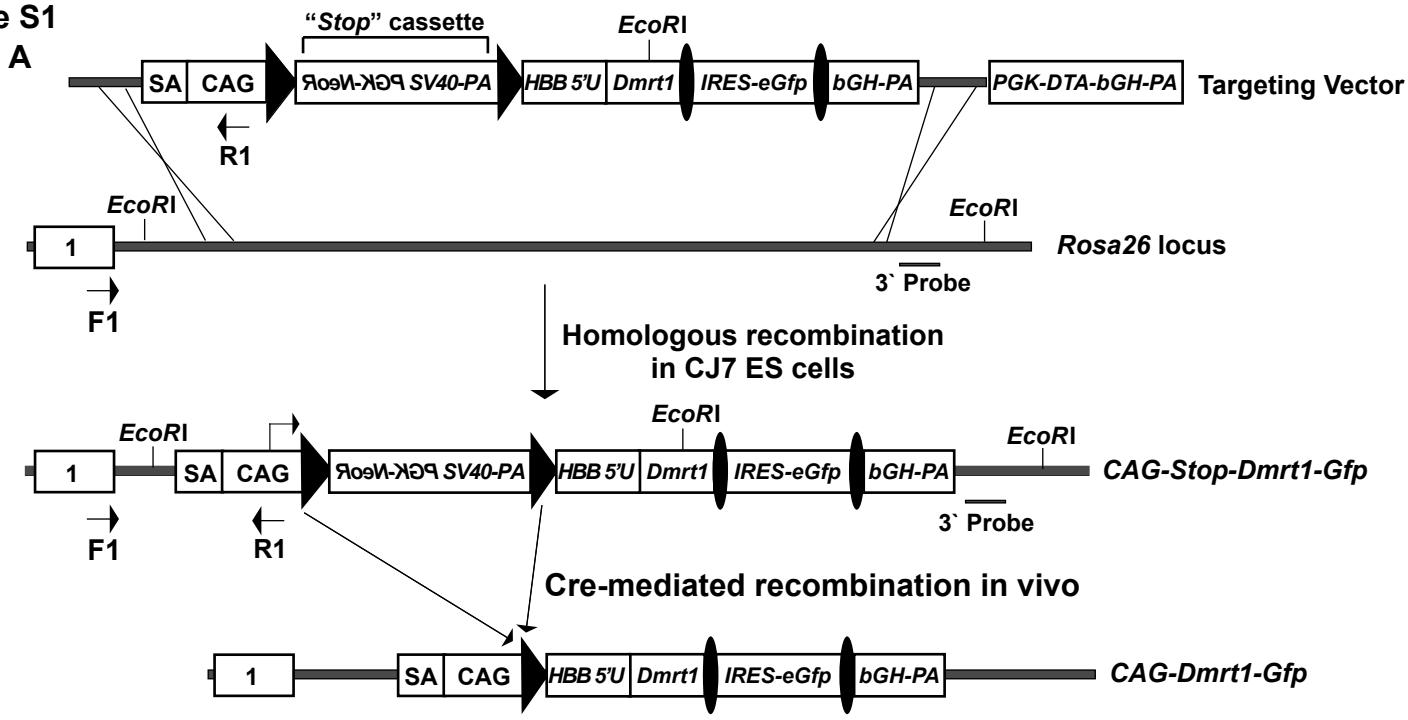
Figure S1


A
"Stop" cassette
EcoRI
SA
CAG
PGK-Neor SV40-PA
HBB 5'U
Dmrt1
IRES-eGfp
bGH-PA
PGK-DTA-bGH-PA
Targeting Vector
R1
EcoRI
EcoRI
1
Rosa26 locus
3` Probe
F1
Homologous recombination
in CJ7 ES cells
EcoRI
EcoRI
EcoRI
1
SA
CAG
PGK-Neor SV40-PA
HBB 5'U
Dmrt1
IRES-eGfp
bGH-PA
CAG-Stop-Dmrt1-Gfp
3` Probe
F1
R1
Cre-mediated recombination in vivo
1
SA
CAG
HBB 5'U
Dmrt1
IRES-eGfp
bGH-PA
CAG-Dmrt1-Gfp
= loxP
= frt


B
5' arm (PCR)
1248 bp
WT
m
1
2
3' arm (Southern)
11 kb
11 kb
9 kb
WT
1
2

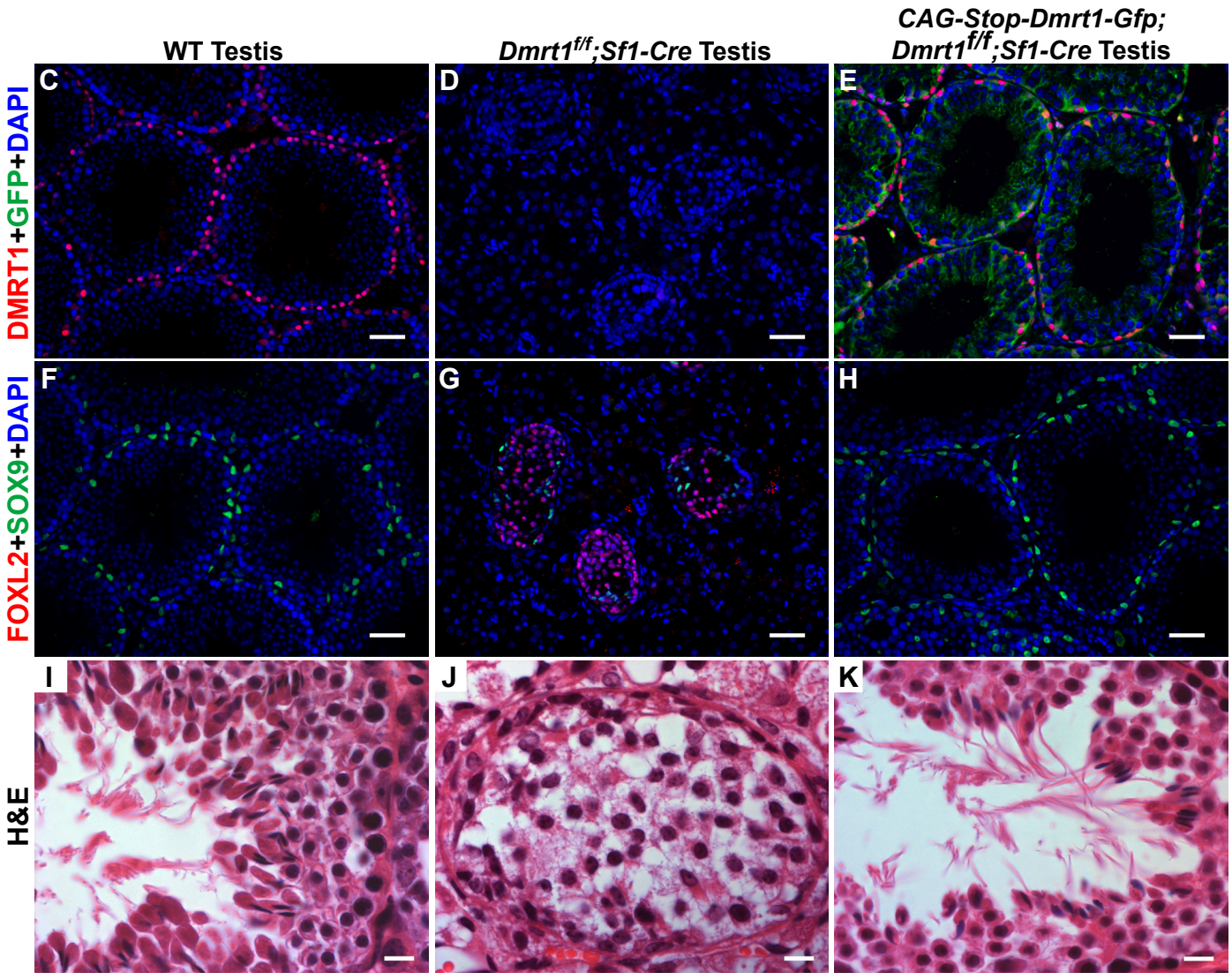

WT Testis
Dmrt1f/f;Sf1-Cre Testis
CAG-Stop-Dmrt1-Gfp;
Dmrt1f/f;Sf1-Cre Testis
DMRT1+GFP+DAPI
FOXL2+SOX9+DAPI
H&E
C
D
E
F
G
H
I
J
K

Figure S2

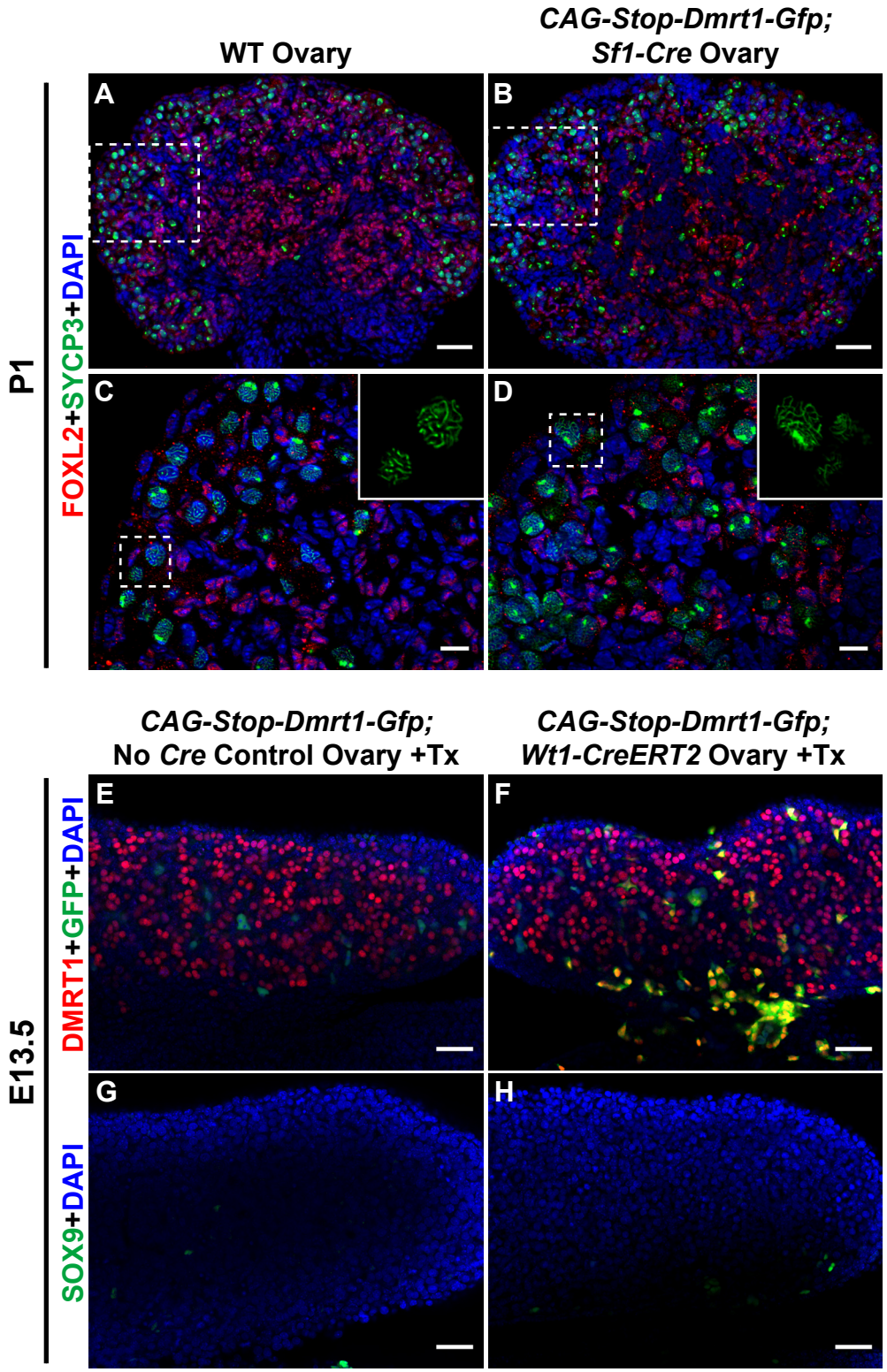
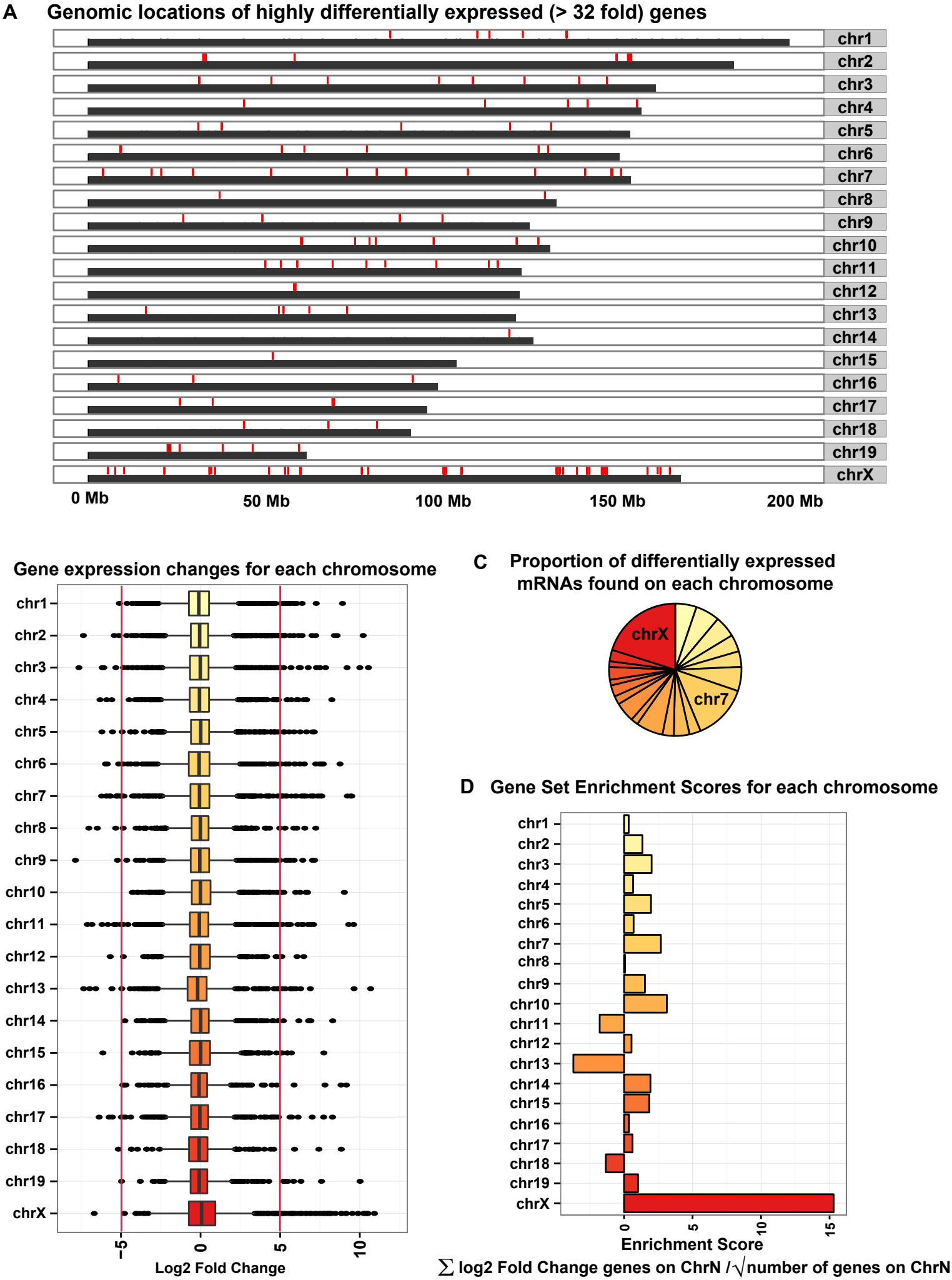

WT Ovary
CAG-Stop-Dmrt1-Gfp; Sf1-Cre Ovary
P1
FOXL2+SYCP3+DAPI
A
B
C
D
CAG-Stop-Dmrt1-Gfp; No Cre Control Ovary +Tx
CAG-Stop-Dmrt1-Gfp; Wt1-CreERT2 Ovary +Tx
E13.5
DMRT1+GFP+DAPI
SOX9+DAPI
E
F
G
H

Figure S3


A
Genomic locations of highly differentially expressed (> 32 fold) genes
chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
chr13
chr14
chr15
chr16
chr17
chr18
chr19
chrX
0 Mb
50 Mb
100 Mb
150 Mb
200 Mb
B
Gene expression changes for each chromosome
chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
chr13
chr14
chr15
chr16
chr17
chr18
chr19
chrX
−5
0
5
10
Log2 Fold Change
C
Proportion of differentially expressed mRNAs found on each chromosome
chrX
chr7
D
Gene Set Enrichment Scores for each chromosome
chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
chr13
chr14
chr15
chr16
chr17
chr18
chr19
chrX
0
5
10
15
Enrichment Score
∑ log2 Fold Change genes on ChrN /√number of genes on ChrN

Supplemental Figure Legends:

Figure S1, related to Figure 1. DMRT1 conditional expression transgene. (A) Targeting vector (top) used to insert conditional expression construct for *Dmrt1* into the *Rosa26* locus in ES cells. Between the two *Rosa26* homology regions, the vector contains a splice acceptor sequence (SA -a cloning remnant, and a synthetic enhancer/promoter/exon/intron/splice acceptor element "CAG promoter" followed by a *loxP*-flanked ("floxed"; black triangles) transcriptional stop cassette containing a reverse-oriented *Pgk-Neo* selectable marker and the *SV40* terminator/polyA sequence. Following the stop cassette is the *Dmrt1* coding sequence preceded by the human *β-globin* (*HBB*) 5' untranslated region (UTR) and followed by a "flrted" (*frt*-flanked; black ovals) internal ribosomal entry site (IRES)-*enhanced green fluorescent protein* (*eGfp*) cassette that can be removed by Flp-mediated recombination, and finally a *bovine growth hormone* 3' UTR/polyA site (*bGH-PA*). The vector also contains a diphtheria toxin negative selection cassette (*PGK-DTA-bGH-PA*) with diphtheria toxin expressed from the *PGK* promoter and followed by a *bGH-PA* site. Homologous recombination into the *Rosa26* locus removed *PGK-DTA-bGH-PA* and produced the single copy transgene *CAG-Stop-Dmrt1-Gfp*. Cre-mediated recombination of *CAG-Stop-Dmrt1-Gfp* deletes the "floxed"stop cassette to generate *CAG-Dmrt1-Gfp*. (B) Correct homologous recombination of the 5' homology arm of the targeting vector into *Rosa26* was confirmed by PCR of ES cell genomic DNA using primers F1 and R1 (indicated in panel A), which amplify between exon 1 of *Rosa26* and the CAG promoter (left panel). No amplification product was detected in wild type

(WT) CJ7 ES cells, but amplicons of the predicted 1248 bp were detected in many targeted ES cell clones (lanes 1 and 2). “m” indicates DNA size markers. Homologous recombination of the 3’ vector arm was confirmed by PCR (not shown) and by Southern blotting using the 3’ probe indicated in panel A. Probing *EcoR*I digested wild type ES cell DNA detected the predicted 11 kb fragment, whereas many targeted clones (e.g. lanes 1 and 2) had both this fragment and a 9 kb fragment corresponding to the targeted allele. Two clones were karyotyped and blastocyst injections were performed using one clone to generate the *CAG-Stop-Dmrt1-Gfp* mice used in this paper. (C-K) Immunofluorescence (IF) and Hematoxylin/eosin (H&E) staining of adult gonads showing rescue of *Dmrt1* null mutation by *CAG-Stop-Dmrt1-gfp*. (C,F,I) WT testis expresses DMRT1 in Sertoli cells and spermatogonia and SOX9 in Sertoli cells does not express FOXL2, and is actively undergoing spermatogenesis. (D,G,J) Conditional deletion of *Dmrt1* in somatic cells of the fetal gonad using *Sf1-Cre* eliminates DMRT1 expression in Sertoli cells and leads to germ cell death. Most mutant Sertoli cells transdifferentiate into FOXL2-positive granulosa-like cells. (E,H,I) Activation of *CAG-Stop-Dmrt1-Gfp* together with deletion of *Dmrt1* using *Sf1-Cre* rescues Sertoli cell differentiation: GFP-positive Sertoli cells expressing DMRT1 and SOX9 are present, no FOXL2 expression is apparent, and spermatogenesis appears normal, with germ cells from spermatogonia to elongated spermatids present. Scale bars C-H: 40 μm, I-K: 10 μm.

Figure S2, related to Figure 2: Early activation of DMRT1 does not masculinize the fetal ovary. (A-D) IF showing that activation of *CAG-Stop-Dmrt1* with *Sf1-Cre* in the fetal gonad does not disrupt specification of oocytes, meiotic initiation, or the normal diplotene arrest (indicated by accumulation of SYCP3 on synapsed chromosomes) at birth. Dashed boxes in A and B indicate areas shown at higher magnification in C and D. Dashed boxes in C and D indicate areas of SYCP3 IF shown in higher magnification insets. (E-H) IF showing that activation of *CAG-Stop-Dmrt1* with *Wt1-CreERT2* at E9.5 results in widespread expression of DMRT1 and GFP in somatic cells by E13.5 but does not activate SOX9. Scale bars A-B,E-H: 40 μm, C-D: 10 μm.

Figure S3, related to Figure 3: Highly differentially expressed genes in the *CAG-Stop-Dmrt1;Sf1-Cre* ovary are disproportionally found on the X chromosome. A) Distribution along chromosomes of genes that were differentially expressed (> 32 fold) in WT versus *CAG-Dmrt1-Gfp* expressing ovaries. Red marks indicate approximate positions of differentially expressed transcripts. B) Box-and-whiskers plot showing distribution of altered gene expression in *CAG-Dmrt1-Gfp* expressing ovaries by chromosome (as $\log_2$-fold changes). Black lines indicate median for each chromosome and shaded boxes enclose interquartile range. Each dot represents a single gene over- or under-expressed more than 1.5-fold outside the interquartile range. Red lines mark 32-fold differential expression. C) Pie chart showing proportion of highly differentially expressed (>32-fold) genes on each chromosome. D) Bar graph showing enrichment score for each chromosome (sum of log-fold changes for all genes

divided by square root of number of genes). The X chromosome shows the highest enrichment score.

Table S1, related to Figure 3: Sheet A) Table of mRNAs differentially expressed ($p<0.05$ and >2-fold) between wild type and *CAG-Dmrt1-Gfp* expressing ovaries. Sheet B) Analysis of the effect of chromosomal location on the number of mRNAs and degree of enrichment in *CAG-Dmrt1-Gfp* expressing ovaries; Analysis of retinoic acid responsive genes in *CAG-Dmrt1-Gfp* expressing ovaries. Genes are classified according to [S1] .Sheet C) Table of mRNAs that exhibit significant differential expression (corrected Z-score > 0.1) between cells identified in the first and last third of pseudotime. Genes that were used for ordering or previously reported [S2] to have dimorphic expression in fetal gonads are indicated.

Supplemental Experimental Procedures:

Mice. *Wt1*$^{tm2(cre/ERT2)Wtp}$ (*Wt1-CreERT2*) [S3] mice were obtained from Jackson Laboratories (Stock Numbers 010912 and 015854). *Sf1-Cre* mice [S4] were provided by K. Parker, *UBC-CreERT2* mice [S5] by E. Brown, *Hsd17b1-CreERT2* mice [S6] by E. Casanova, *Sox9*$^{flox/+}$ mice [S7] by R. Behringer, and *Sox8*$^{+/-}$*;Sox9*$^{flox/+}$ mice [S8] by M. Wegner. *Dmrt1*$^{flox/+}$ mice are described in [S9]. Mice were of mixed genetic background (129Sv and C57Bl/6J). Presence of a copulation plug in the morning was recorded as day E0.5. Experimental protocols were approved by the University of Minnesota Animal Care and Use Committee.

Genotyping. PCR genotyping on tail clip DNA for the *CAG-Stop-Dmrt1-Gfp* transgene was conducted using primers R26-F (5' GCTCTCCCAAAGTCGCTCTGAG 3'), ADK5-R (5' GCCCCAGCTACAGCCTCGATTTGTG 3') and CMV-R (3' GGAAAGTCCCTATTGGCGTT), which yield an approximately 850 bp band for the WT allele and a 500 bp band for the transgenic allele. Reactions ran for 35 cycles with a 60^{o} annealing step and a 1 min 72^{o} elongation. PCR conditions and primers for genotyping the *Dmrt1* floxed allele and *Cre* transgenes are described in [S9].

Inducible DMRT1 knock-in allele. The inducible *Dmrt1* vector was generated by inserting a fragment containing the human beta-globin (HBB) 5'UTR followed by the mouse *Dmrt1* coding sequence into the AscI site of the CAG-STOP-eGFP-ROSA26TV (CTV) vector [S10]. This vector was inserted into the *Rosa26* locus by homologous recombination in CJ7 ES cells as diagrammed in Figure S1.

Tamoxifen-induced activation of *CAG-Stop-Dmrt1-Gfp* transgene. Stock solutions were prepared by dissolving 100 mg of tamoxifen (Sigma) in 5 ml sesame oil (Sigma) to 20 mg/ml. For adult induction, 100 μl of tamoxifen solution (2 mg per mouse) was injected IP once a day for 5 days. Gonads were harvested 3 days (*UBC-Cre*) or 14 days (*Hsd17b1-Cre*) after the end of the injection time course. For *Wt1-CreERT2* embryonic induction, timed matings were set up and pregnant females were IP injected with 100 ul of 20mg/ml tamoxifen once at E8.5. Embryonic gonads were then harvested at E10.5, E11.5, or E13.5.

Immunofluorescence. Immunofluorescence was performed as previously described [S11], with antibodies listed in [S12] and [S13]. The rabbit anti-SOX9-CT antibody used in the *Sox9* KO experiments was a gift from F. Poulat and is described in [S14]. Images were captured with a Zeiss Imager Z1 microscope and Zeiss MRm camera. Images were processed and false-colored using Zeiss Axiovision software and further processed in Adobe Photoshop. Higher magnification images were captured using Zeiss Apotome structured illumination. Whole mount immunostaining on embryonic gonads was performed as in [S15] and imaged on a Zeiss LSM710 confocal microscope using the Zen software package.

RNA analysis. RNA was prepared in duplicate from gonads of 8-10 week-old WT male, WT female and *CAG-Stop-Dmrt1-Gfp;Sf1-Cre* mice on a mixed background. Short-insert libraries were prepared from polyA+ mRNA using Illumina TruSeq v3 and ~35 million 50bp paired-end reads were obtained for each sample on an Illumina HiSeq 2000. Reads were mapped to mm9 and counts were assigned to genes defined by the Ensembl release 67 annotation. Differentially expressed genes were identified with DESeq2. For single cell RNA-Seq, *CAG-Stop-Dmrt1-Gfp;Sf1-Cre* ovaries at P17 were dissociated and ~0.5 million cells were FACS sorted based on forward- and side-scatter, GFP and the Zombie Aqua viability stain (Biolegend). Cells were captured with a 5-10μm cell capture cassette using the Fluidigm C1 instrument. cDNA was synthesized and amplified using SMARTer-Seq (ClonTech). Of the 89 captured cells, 68 were selected for further analysis based on post-capture imaging and cDNA yields.

Libraries were created using the Nextera XT (Illumina) protocol. MiSeq (Paired-End 75bp) and HiSeq 2000 (Single-Read 50bp) data were trimmed and mapped to mm9 as above. Normalized FPKM values were exported to Monocle to construct pseudotemporal ordering. Count data from cells assigned to the first and last third of pseudotime was analyzed with SCDE. RNA-seq data are available from GEO (accession number GSE64960). Details of the bioinformatics analysis can be found at https://github.com/micahgearhart/cag-dmrt1.

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