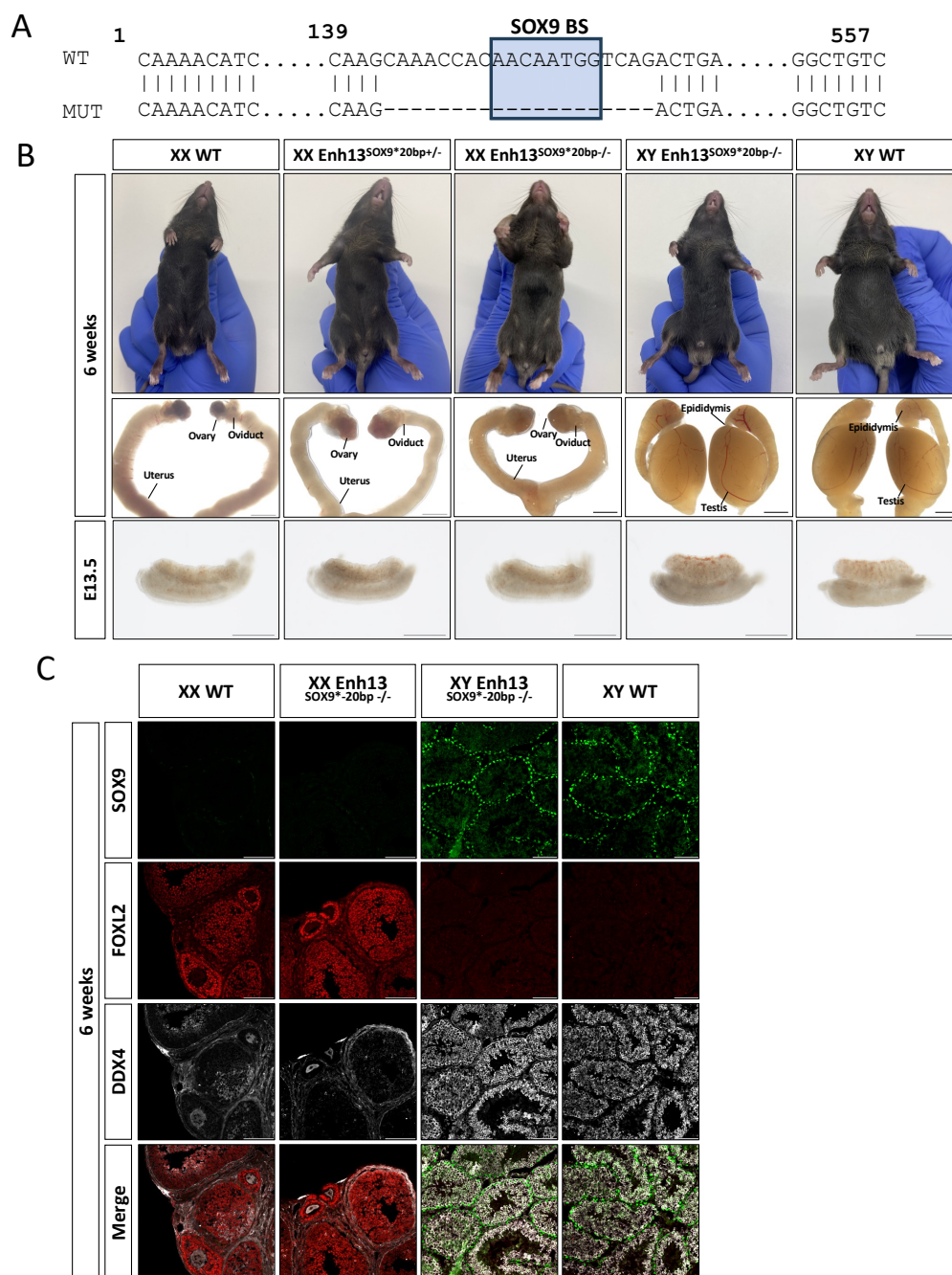


Supplementary Figures and Tables

Supplementary Figures 1 to 12

Supplementary Tables 1 to 4

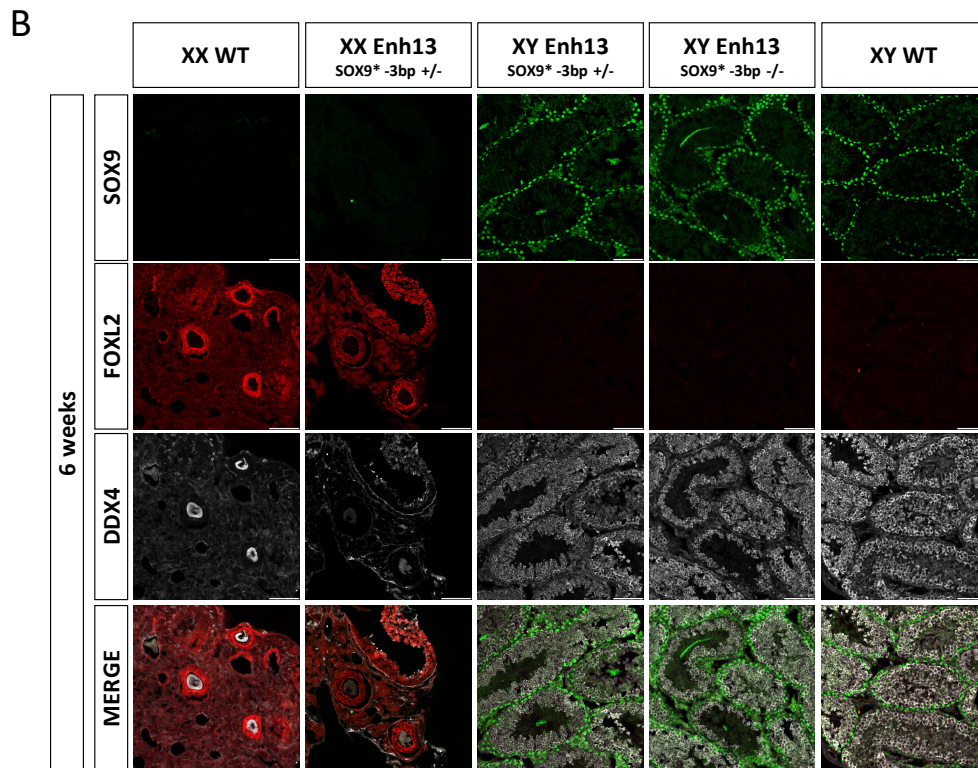
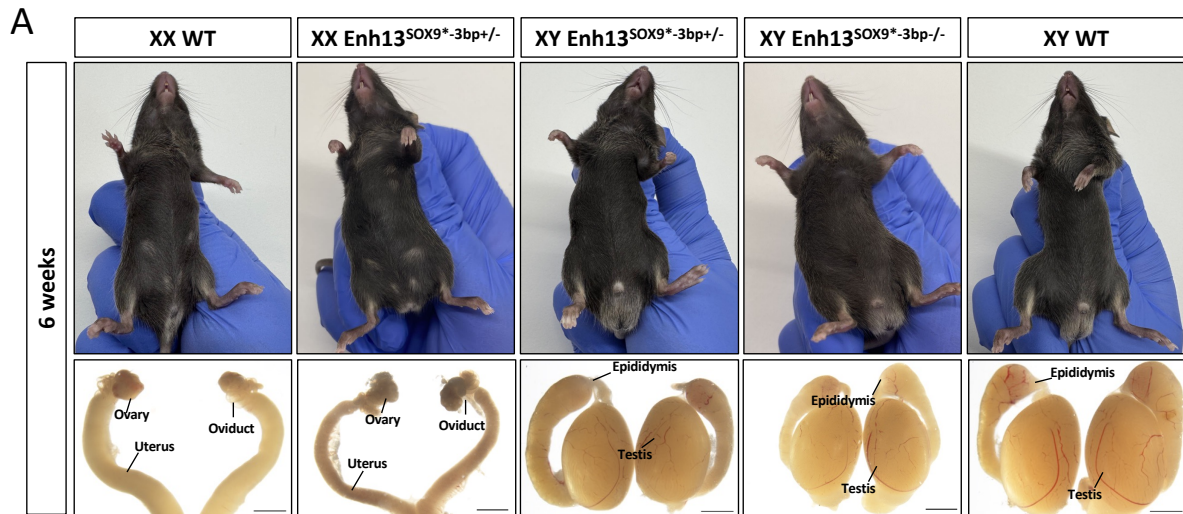
Supplementary Data 1-2



Supplementary Figure 1. Characterization of mouse strain containing a 20bp deletion in the SOX9 BS of Enh13 (Enh13^{SOX9*-20bp})

(A) Sequence representation and representative alignment between WT sequence and homozygous mutation of mouse line containing 20bp deletion around the SOX9 BS of Enh13 (Enh13^{SOX9*-20bp})

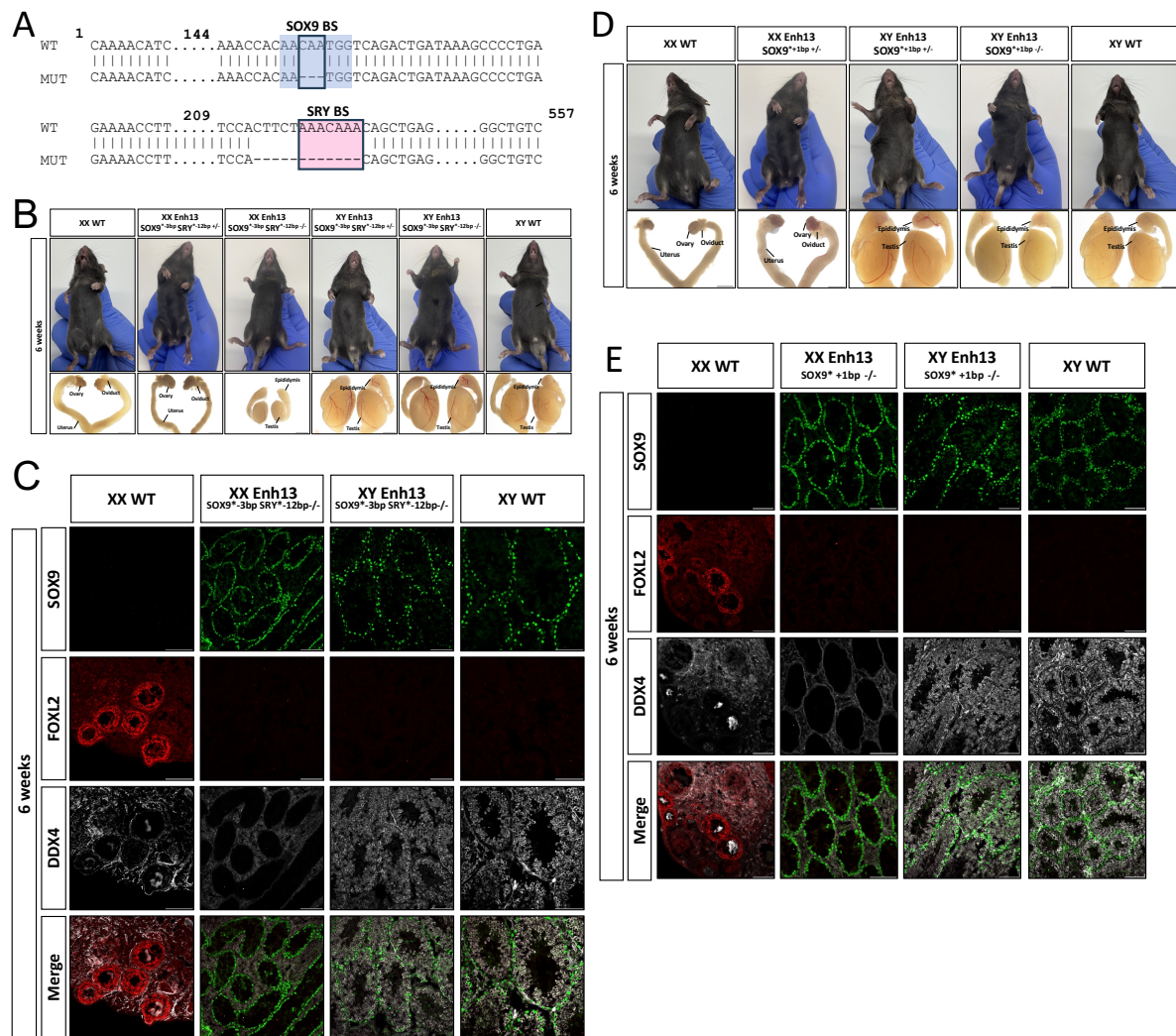
using the online alignment tool - BLAST. SOX9 BS is highlighted in blue. (B) Bright field images of the external and internal genitalia, and gonads of 6-week-old adult mice. Scale bar represents 2000 μm . (C) Immunostaining of 6-week-old gonads from XX WT, XX Enh13^{SOX9*-20bp-/-}, XY Enh13^{SOX9*-20bp-/-} and XY WT. Gonads were stained for Sertoli-marker SOX9 (green), granulosa-marker FOXL2 (red) and germ cell marker DDX4 (grey). Scale bars represent 100 μm . WT- *Wild Type*.



Supplementary Figure 2. Characterization of mouse strain containing a 3bp deletion in the SOX9 BS of Enh13 (Enh13^{SOX9* -3bp})

(A) Bright field images of the external and internal genitalia, and gonads of XX WT, XX Enh13^{SOX9* -3bp +/-}, XY Enh13^{SOX9* -3bp +/-}, XY Enh13^{SOX9* -3bp -/-} and XY WT of 6-week-old adult mice. Scale bar represents 2000 μ m (B) Immunostaining of 6-week-old gonads of XX WT, XX Enh13

SOX9^{*-3bp+/-}, XY Enh13^{SOX9^{*-3bp+/-}}, XY Enh13^{SOX9^{*-3bp-/-}} and XY WT. Gonads were stained for Sertoli-marker SOX9 (green), granulosa-marker FOXL2 (red) and germ cell marker DDX4 (grey). Scale bars represent 100 μ m. WT- *Wild Type*.



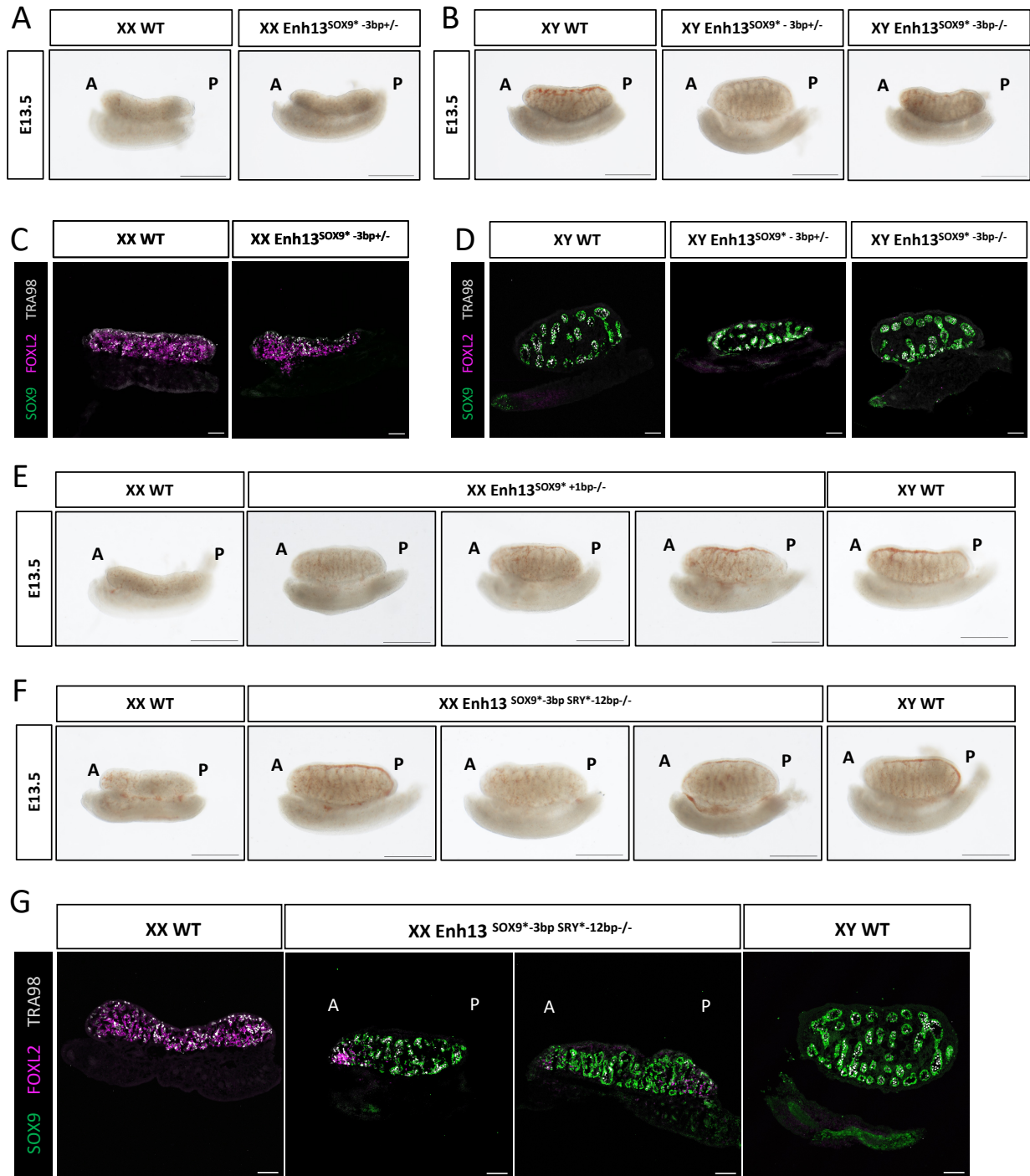
Supplementary Figure 3. Characterization of mouse strains containing a 3 bp deletion on the SOX9 BS as well as 12 bp deletion in the SRY BS or 1bp insertion in the SOX9 BS of Enh13

(A) Sequence representation and representative alignment between WT sequence and homozygous mutation Enh13^{SOX9^{+3bp} SRY^{+12bp}} mutants using the online alignment tool - BLAST. SOX9 BS is highlighted in blue. SRY BS is highlighted in pink. WT- *Wild Type*.

(B, D) Bright field images of the external and internal genitalia, and gonads of XX WT, XY WT and Enh13^{SOX9^{+3bp} SRY^{+12bp}} mutants (B) or Enh13^{SOX9^{+1bp}} mutants (D) in 6-week-old adult mice. Scale bar represents 2000 μ m (C, E) Immunostaining of 6-week-old gonads of XY WT, XY WT and Enh13^{SOX9^{+3bp} SRY^{+12bp}} mutants (C) or Enh13^{SOX9^{+1bp}} mutants (E). Gonads were stained for

Sertoli-marker SOX9 (green), granulosa-marker FOXL2 (red) and germ cell marker DDX4 (grey).

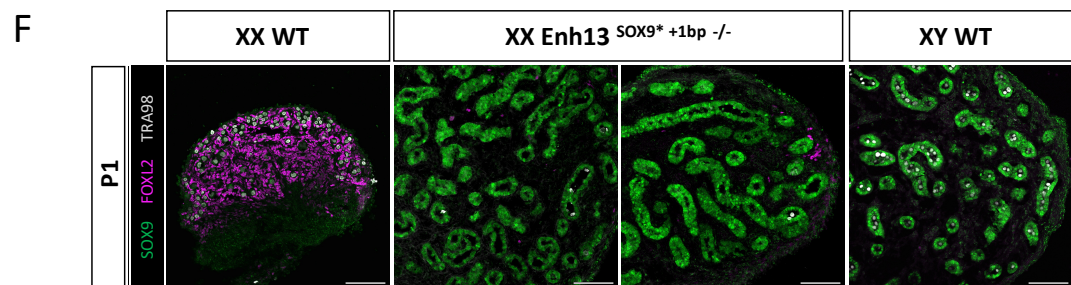
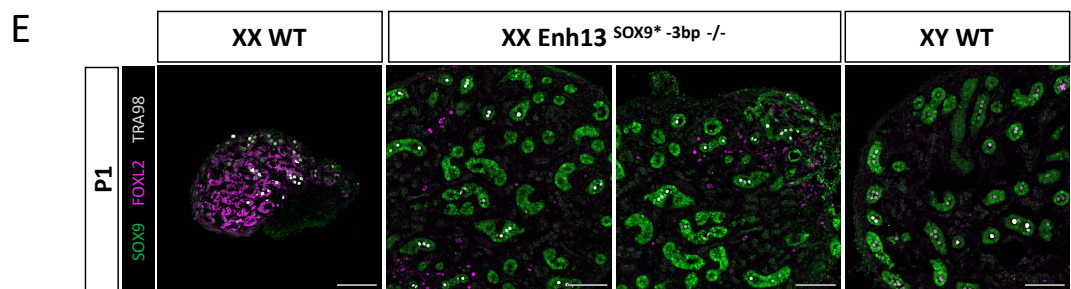
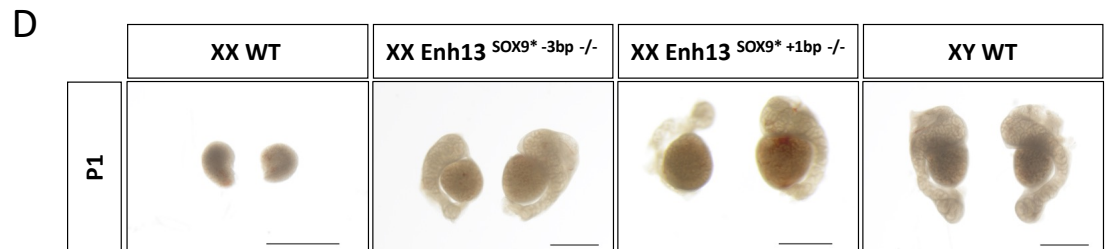
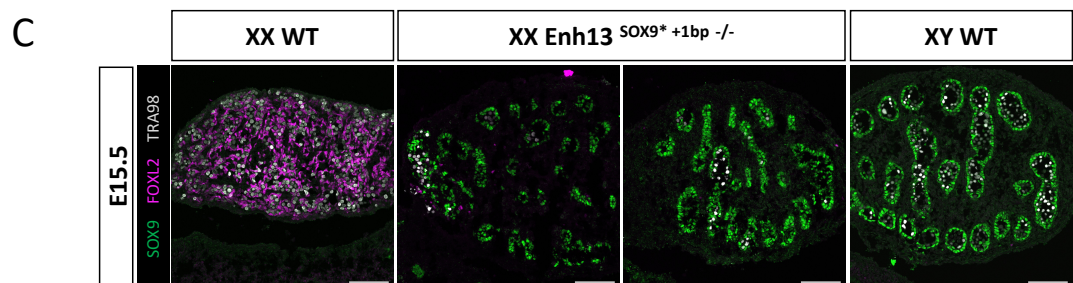
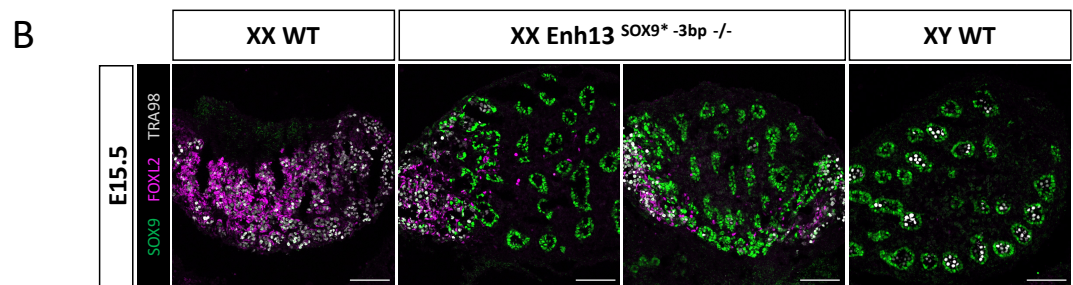
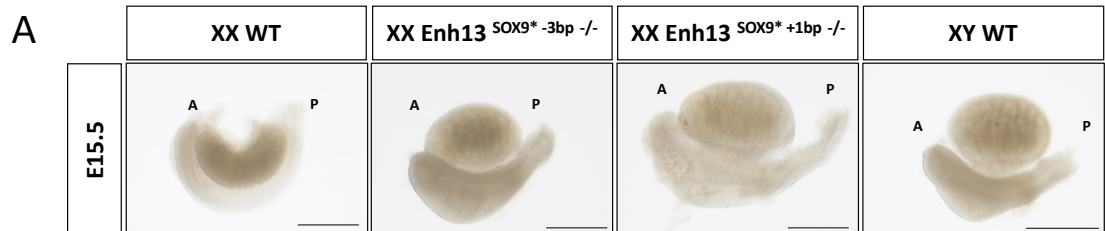
Scale bars represent 100 μm .



Supplementary Figure 4. Characterization of E13.5 embryonic gonads of the various mutant mice strains

(A-B, E-F) Bright field images of E13.5 gonads. Scale Bar represents 500 μ m. (A) Bright field images of E13.5 XX WT and XX Enh13^{SOX9* -3bp+/-} gonads. (B) Bright field images of E13.5 XY

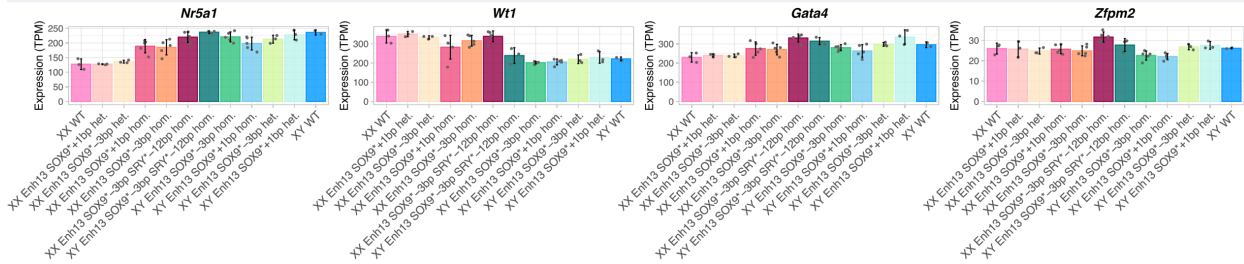
WT, XY Enh13^{SOX9*-3bp+/-}, XY Enh13^{SOX9*-3bp-/-} gonads. (C-D, G) Immunostaining of E13.5 gonads, stained for Sertoli-marker SOX9 (green), granulosa-marker FOXL2 (magenta) and germ cell marker TRA98 (grey). Scale Bar represents 100 μ m. (C) Immunostaining of E13.5 XX WT and XX Enh13^{SOX9*-3bp+/-} gonads. (D) Immunostaining of E13.5 XY WT, XY Enh13^{SOX9*-3bp+/-} and XY Enh13^{SOX9*-3bp-/-} gonads. (E) Bright field images of the E13.5 gonads of XX WT, XX Enh13^{SOX9*+1bp-/-} and XY WT. (F) Bright field images of E13.5 gonads of XX WT, XX Enh13^{SOX9*-3bp SRY*-12bp-/-} and XY WT. (G) Immunostaining of E13.5 gonads of XX WT, XX Enh13^{SOX9*-3bp SRY*-12bp-/-} and XY WT. WT- *Wild Type*. A-anterior, P- posterior.



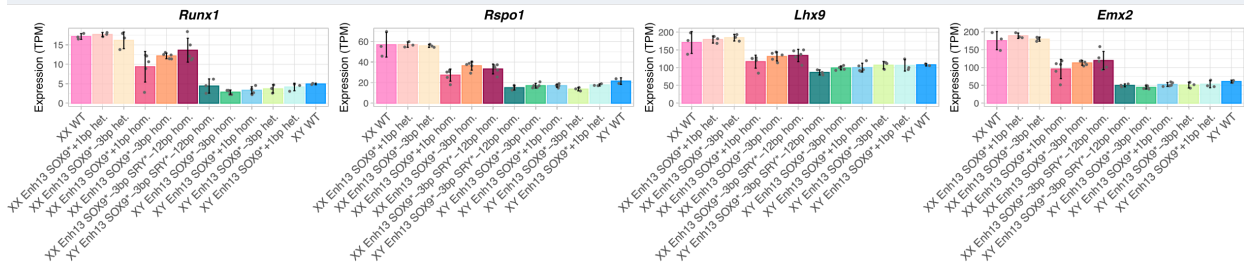
Supplementary Figure 5. Characterization of E15.5 embryonic gonads and P1 gonads of the various mutant mice strains

(A,D) Bright field images of gonads of XX WT, XX Enh13^{SOX9*⁻3bp^{-/-}}, XX Enh13^{SOX9*⁺1bp^{-/-}}, and XY WT gonads at E15.5 (A) and at P1 (D). Scale Bar represents 500 μ m. (B-C, E-F) Immunostaining of gonads stained for Sertoli-marker SOX9 (green), granulosa-marker FOXL2 (magenta) and germ cell marker TRA98 (grey). Scale Bar represents 100 μ m. (B, E) Immunostaining of E15.5 (B) or P1 (E) gonads of XX WT, XX Enh13^{SOX9*⁻3bp^{-/-}}, and XY WT. (C, F) Immunostaining of E15.5 (C) or P1 (F) gonads of XX WT, XX Enh13^{SOX9*⁺1bp^{-/-}}, and XY WT WT- *Wild Type*. A-anterior. P- posterior.

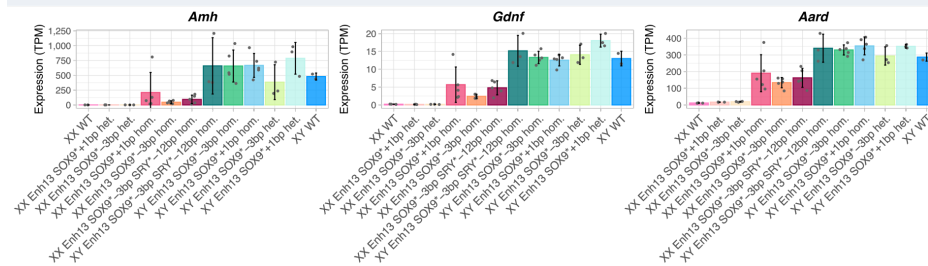
A Somatic cells



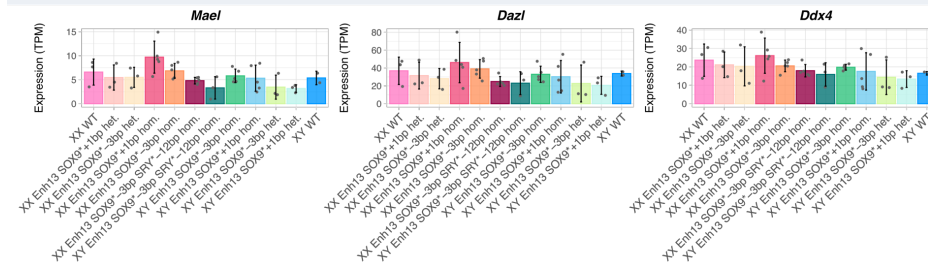
B Pre-granulosa cells



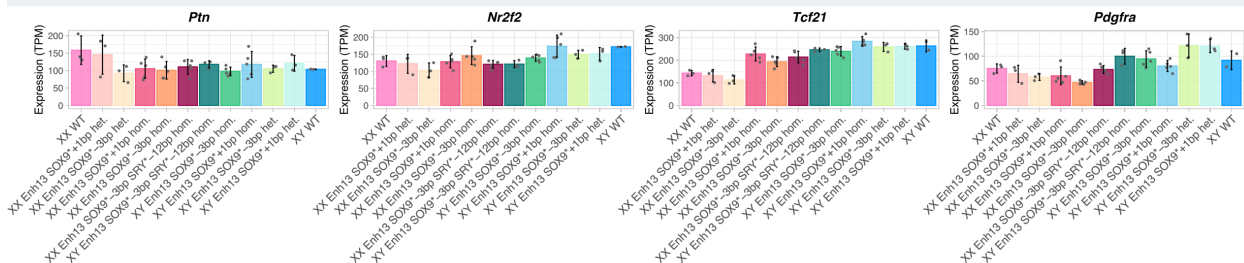
C Sertoli cells



D Germ cells



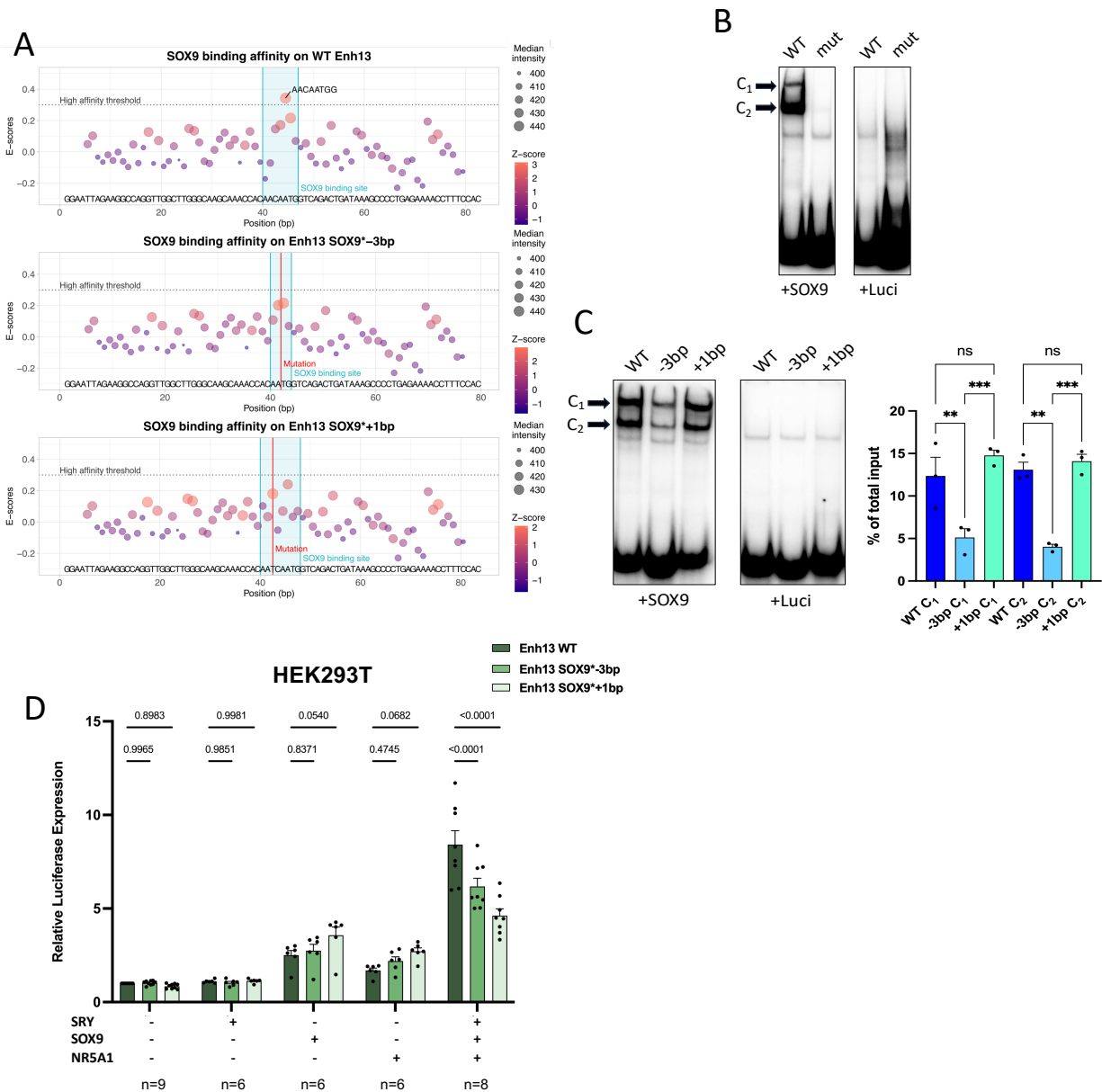
E Interstitium/Stroma cells



Supplementary Figure 7. Expression of gonadal cell markers at E12.5 WT and mutant gonads

Expression profiles of well-known gonadal marker genes for somatic cells (A), pre-granulosa cells (B), Sertoli cells (C), germ cells (D) and Interstitial cells (E). Expression values are expressed as

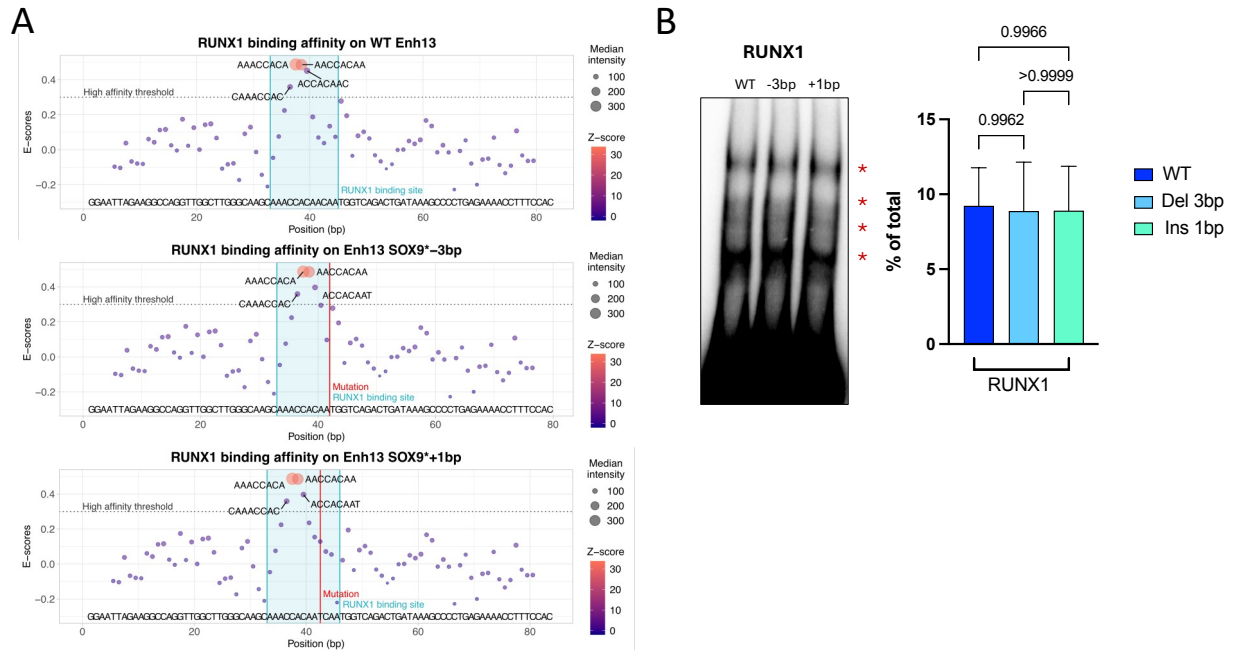
TPM (Transcript per million). Bars represent the median of expression, while each point represent a single biological replicate. Error bars represent the standard deviation across the replicates.



Supplementary Figure 8. Exploration of SOX9 binding affinity to WT and mutated Enh13 sequences

(A) Visualization of the SOX9 binding affinity for the WT and the mutated Enh13 sequences around the mutation area as measured using PBM. The dash line represents the threshold from which the affinity is considered as high. The size of the dot represents the median intensity signal of the microarray for each 8-mers composing the sequence. The dots are coloured by the z-score of the intensity signal of each 8-mers compared to the rest of the sequences tested on the microarray. (B) EMSA using short probes containing SOX9 BS region in Enh13 WT, and Enh13

mutated sequence is which the SOX9 BS is abolished^{35, 36}. Probes were loaded in the presence of SOX9 protein (left) or Luciferase protein (right). Arrow highlights the complexes formed with the shifted probe due to binding in the presence of SOX9 protein. No complexes are detected in the mutated Enh13 sequence. (C) EMSA using short probes containing SOX9 BS region in Enh13 WT, Enh13 3bp deletion, and Enh13 1bp insertion. Probes were loaded in the presence of SOX9 protein (left) or Luciferase protein (right). Arrow highlights the complexes formed with the shifted probe due to binding in the presence of SOX9 protein. Bar graph shows the quantification of the binding complexes. (D) Luciferase reporter assays of HEK293T cells, co-transfected with SRY, SOX9, and NR5A1 expression plasmids or each factor alone, along with Luciferase reporter plasmid containing the sequences of Enh13 WT, Enh13^{SOX9*-3bp}, or Enh13^{SOX9*+1bp}. All values were normalized to the Enh13 WT Luciferase activity in the absence of expression plasmids. Sample size for each condition is indicated on the graph where each dot represents an independent experiment. Samples were compared to the Enh13 WT plasmid within each condition. Means and SEM from at least three independent experiments are shown. Statistical analysis was done using two-way ANOVA followed by Tukey's/Dunnett's posttest. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, ns- not significant. WT- *Wild Type*. Source data are provided as a Source Data file.



Supplementary Figure 9. Exploration of RUNX1 binding affinity to WT and mutated Enh13 sequences

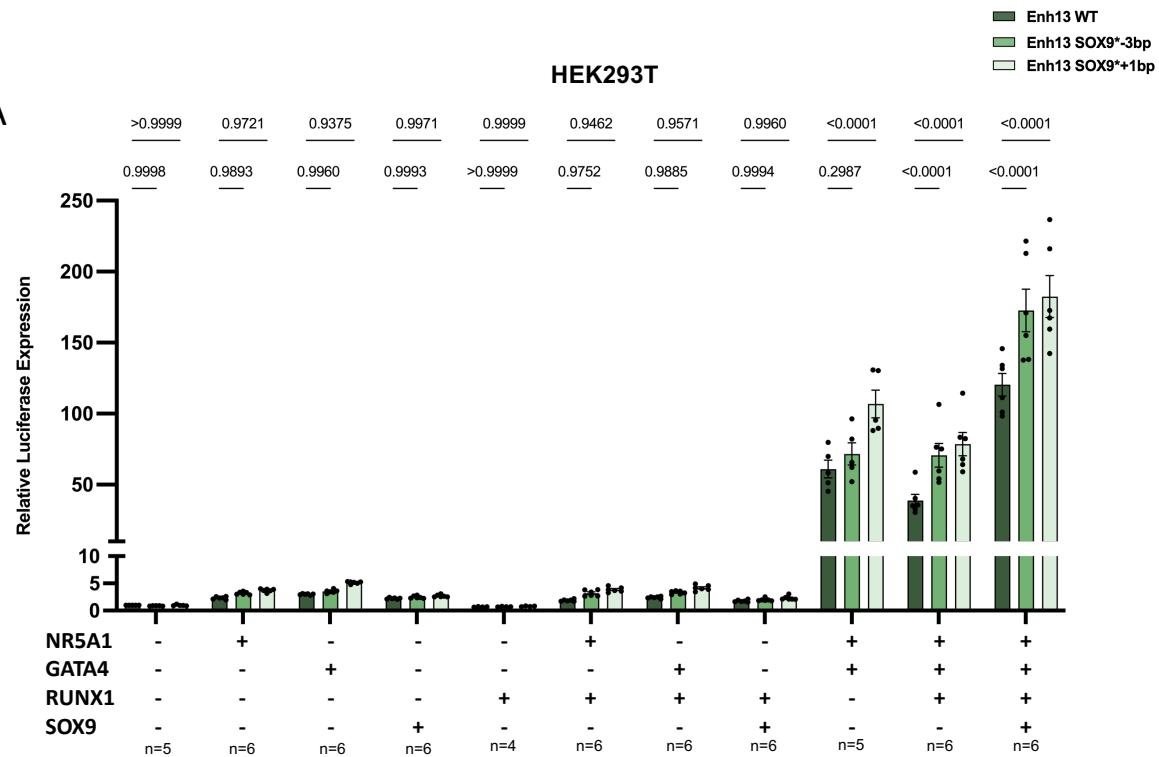
(A) Visualization of the RUNX1 binding affinity for the WT and the mutated Enh13 sequences around the mutation area measured using PBM. The dash line represents the threshold from which the affinity is considered as high. The size of the dot represents the median intensity signal of the microarray for each 8-mers composing the sequence. The dots are coloured by the z-score of the intensity signal of each 8-mers compared to the rest of the sequences tested on the microarray. (B) EMSA using long probes containing the region presented in Fig. 4A of Enh13 WT, Enh13^{SOX9*-3bp}, and Enh13^{SOX9*+1bp}. Probes were loaded in the presence of RUNX1 protein. Asterisks highlight the complexes formed with shifted probe due to binding in the presence of RUNX1 protein. Bar graph shows the quantification of the binding complexes. WT- *Wild Type*. Source data are provided as a Source Data file.

A. Enh13 WT

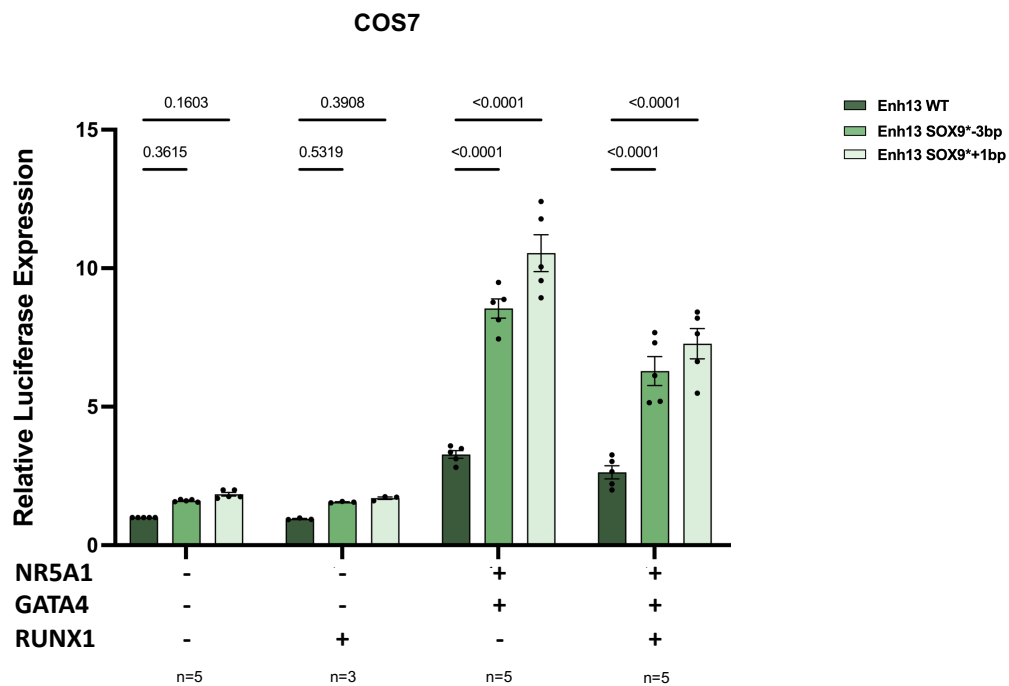
[illegible]

Supplementary Figure 10 Putative Transcription Factor Binding Sites analysis in Enh13 and mutated Enh13 sequences (A-C) Visualization of putative binding sites analysis using JASPAR dataset of Enh13 sequence for well-known TFs expressed in the gonad. Coloured boxes represent putative TFBS with high affinity scores. Grey box highlights the SOX9 binding site region in Enh13 where the 3 bp del or 1 bp insertion reside. (A) JASPAR analysis of the Enh13 WT sequence (B) JASPAR analysis of the Enh13 ^{SOX9*-3bp} sequence (C) JASPAR analysis of the Enh13 ^{SOX9*+1bp} sequence.

A

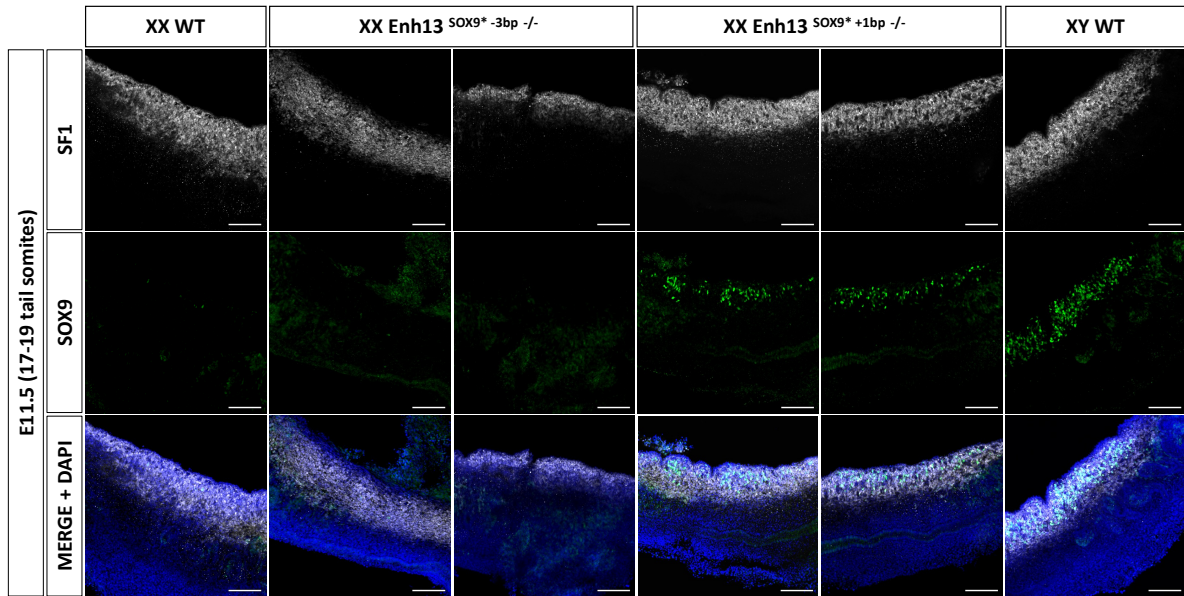


B



Supplementary Figure 11. Luciferase assays mimicking TF present at the bipotential gonad
(A-B) Luciferase reporter assays performed in HEK293T cells (A) or COS7 cells (B), co-transfected with combinations of expression plasmids of TFs with binding sites near the SOX9 BS (RUNX1, NR5A1 and GATA4) along with Luciferase reporter plasmid containing the sequences of Enh13 WT, Enh13^{SOX9*-3bp}, or Enh13^{SOX9*+1bp}. All values were normalized to the Enh13 WT Luciferase activity in the absence of expression plasmids. Sample size for each condition is indicated on the graph where each dot represents an independent experiment. Samples were compared to the Enh13 WT plasmid within each condition. Means and SEM from at least three independent experiments are shown. Statistical analysis was done using a two-way ANOVA followed by Tukey's/Dunnett's posttest. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, ns- not significant. Source data are provided as a Source Data file.

A



Supplementary Figure 12. Characterization of E11.5 embryonic gonads of the various mutant mice strains

(A) Whole-mount immunostaining of E11.5 XX WT, XX Enh13^{SOX9* -3bp -/-}, XX Enh13^{SOX9* +1bp -/-}, and XY WT gonads stained for somatic cell marker SF1 (grey) and Sertoli-marker SOX9 (green). Scale Bar represents 100 μ m.

Table S1. Primers for genotyping mice and cloning				
Target	Primer name	Description	Sequence 5' to 3'	Product and size
Sex	Sex_F	X/Y chromosome	5'-GATGATTTGAGTGGAATGTGAGGTA-3'	Sex_F + Sex_R: 280bp in XY; 685bp,660bp and 480bp in XX (McFarlane et al., 2013)
	Sex_R	X/Y chromosome	5'-CTTATGTTTATAGGCATGCACCATGTA-3'	
Enh13 616bp	Enh13_F3	Flanks 5' end of Enh13	5'-TTAAGAAGGTAGACCCAGAG-3'	Enh13_F3 + Enh13_R4: 616bp in WT allele or INDELS; 412bp in deleted alleles (204 or 210 bp del)
	Enh13_R4	Flanks 3' end of Enh13	5'-CCTCATTCTTTGCAGAAAGC-3'	
Enh13 557bp	Enh13_BglII_F	Flanks 5' end of Enh13 and adds BglII site	5'-CAGAGATCTCAAAACATCCAGGTGGG-3'	Amplified 557bp of Enh13 while adding restriction enzyme sites for BglII and HindIII
	Enh13_HindIII_R	Flanks 3' end of Enh13 and adds HindIII site	5'-CTCAAGCTTGACAGCCACATGCAATC-3'	
PGL4.26 MCS	RV3	Sits upstream to the MCS in PGL4.26	5'CTAGCAAAATAGGCTGTCCC-3'	Primer used for sequencing the PGL4.26 plasmid

Table S2. sgRNA used for CRISPR genome editing			
Application	sgRNA name	Sequence 5' to 3'	PAM
Enh13_SOX9_BS sgRNA	SOX9_BS_G1	TATCAGTCTGACCATTGTTG	TGG
Enh13_SRY_BS sgRNA	SRY_BS_G1	CTCAGCTGTTTGTTTAGAAG	TGG

Table S3. Antibodies/ Dyes used in this study								
Target	Host	Manufacture	Cat. number	Lot number	Dilution	Antibody type	Used for staining	Cell type
αSOX9	Goat	R&D systems	AF3075	WL0523091	1:300	Primary	Gonads (E11.5, E13.5, E15.5, P1, 6W)	Sertoli cells
αFOXL2	Rabbit	Abcam	ab246511	1067642-41	1:300	Primary	Gonads (E13.5, E15.5, P1, 6W)	Granulosa cells
αDDX4	Mouse	Abcam	ab27591	1067533-24	1:300 for males / 1:2000 for females	Primary	Adult gonads (6W)	Germ cells
αGCNA1 (TRA98)	Rat	Abcam	ab82527	1098726-9	1:200	Primary	Gonads (E13.5, E15.5, P1)	Germ cells
αNr5a1 (Ad4BP/SF-1)	Rat	Cosmo Bio	KAL-KO610	1B1F10	1:300	Secondary	Embryonic gonads (E11.5)	Somatic cells
αGoat-Alexa flour 488	Donkey	Invitrogen	A-11055		1:500	Secondary	Gonads (E11.5, E13.5, E15.5, P1, 6W)	-
αRabbit-Alexa flour 568	Donkey	Invitrogen	A-10042		1:500	Secondary	Embryonic gonads (E13.5) and Adult gonads (6W)	-
αMouse-Alexa flour 647	Donkey	Invitrogen	ab150111		1:500	Secondary	Adult gonads (6W)	-
αRat-Alexa flour 647	Donkey	Abcam	ab150155		1:500	Secondary	Gonads (E11.5, E13.5, E15.5, P1)	-
DAPI	-	Invitrogen	D-1306		300nM	Dye	All	Nucleic acid

Table S4. Primers used for quantitative RT-PCR			
Primer name	Gene	Marker of	Sequence 5' to 3'
Sox9_F	Sox9	Sertoli cells	AAGAAAGACCACCCCGATTACA
Sox9_R			CAGCGCCTTGAAGATAGCATT
Sox8_F	Sox8	Sertoli cells	AGCGAGAAGAGGCCGTTTG
Sox8_R			TCAGTACCAGAGTCTGAGTCG
Foxl2_F	Foxl2	Granulosa cells	CGGCATCTACCAGTACATCATAGC
Foxl2_R			GCACTCGTTGAGGCTGAGGTTG
Hptr_F	Hprt	House keeping gene	GCTTGCTGGTGA AAAGGACCTCTCGAAG
Hptr_R			CCCTGAAGTACTCATTATAGTCAAGGGCAT