Supplementary Materials for

The histone demethylase KDM6B regulates temperature-dependent sex determination in a turtle species

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Published 11 May 2018, *Science* **360**, 645 (2018)
DOI: 10.1126/science.aap8328

This PDF file includes:

Materials and Methods
Figs. S1 to S18
Tables S1 and S2
References

Materials and Methods

Turtle eggs

All animal experiments were carried out under the animal ethical guideline of Duke University and Zhejiang Wanli University. Freshly laid red-eared slider turtle (*T. scripta*) eggs were obtained from Tangi Turtle Farm (Hammond, LA) and Hanshou Institute of Turtles (Hunan, China). Fertilized eggs were randomized in trays of moist vermiculite and placed in incubators at 26°C (male-producing temperature) or 32°C (female-producing temperature), with humidity maintained at 70-80%. In this species, incubation of eggs at 26°C produces all males, whereas incubation at 32°C generates all females. For temperature shift experiments, 100 eggs were shifted at developmental stage 16 from an incubator kept at 26°C to an incubator kept at 32°C and vice versa. The temperature-sensitive period in *T. scripta* extends from approximately stage 15 to stage 20, when the embryo is environmentally sensitive and when sex determination occurs (21). Embryos were staged according to criteria established by Greenbaum (22). At stages 13-21, 23 and 25, embryos incubated at different temperatures were removed from eggshells, decapitated and placed in PBS for dissection.

RNA extraction and qRT-PCR

Gonads from embryos in each group were microdissected from the mesonephros, and individual pairs of gonads were harvested for RNA extraction using TRIzol (Invitrogen) or RNeasy Plus Micro kit (Qiagen). The cDNA was generated from 0.5-2 μg RNA using the SuperScript First-Strand Synthesis System (Fermantas, USA) based upon the manufacturer's protocol, followed by DNase treatment. Real-time PCR was carried out in triplicate with a SYBR Green Supermix (Bio-Rad) in a Bio-Rad iCycler system. After normalization with *Gapdh*, relative RNA levels in samples were calculated by the comparative threshold cycle (Ct) method. The sequences of primers for qRT-PCR are listed in Table S2.

FPKM values for histone demethylases

Expression data was extracted from the timecourse transcriptome analysis and normalized as in Czerwinski *et al* (17).

Fluorescence in situ hybridization

Gonad-mesonephros complexes dissected from turtle embryos of stage 19 were fixed in 4% PFA overnight at 4°C, then embedded in paraffin wax and sectioned. *In situ* hybridization of sections was carried out as previously described (18). Briefly, antisense riboprobes synthesized from the cDNA fragment of *Kdm6b* were labeled with Digoxigenin(DIG). FITC-DIG antibodies (Roche) were used for signal detection. Nuclei were stained with DAPI. For double staining, the in situ hybridized sections were incubated with VASA antibody, and AlexFluor 594 donkey anti-rabbit IgG was used for detection. Gonadal sections were observed under a fluorescence microscope (Ti-E, Nikon).

Immunofluorescence

Gonad-mesonephros complexes from turtle embryos of indicated stages were fixed in 4% paraformaldehyde, subsequently frozen in OCT embedding medium, stored at -80°C and sectioned at 12 μm. Cryosections were covered with primary antibodies and incubated

overnight at 4°C. The primary antibodies used in this analysis included rabbit anti-DMRT1, (produced privately through Sangon Biotech, 1:250), rabbit anti-KDM6B (produced privately through Sangon Biotech, 1:200, in this case plus permeabilization buffer from BioGems), rabbit anti-SOX9 (Chemicon, AB5535, 1:1000), mouse anti-β-catenin (Sigma, C7207, 1:250), rabbit anti-aromatase (Abcam, ab18995, 1:150), rabbit anti-VASA (Abcam, ab13840, 1:100) and rabbit anti-SCP3 (Novus Biologicals, NB300-231, 1:100). Primary antibodies were detected using secondary antibodies (AlexFluor 488 donkey anti-mouse IgG, and AlexFluor 594 donkey anti-rabbit IgG, both diluted at 1:250). Nuclei were stained with Dapi. Gonad sections were imaged under confocal microscope (Zeiss, LSM710, or Nikon, A1 Plus).

The percentage of H3K27me3 positive cells was determined as the number of H3K27me3-labeled cells from gonadal fields in five randomly selected sections relative to the total number of cells per field.

Aromatase inhibitor and estrogen treatments

A non-steroidal aromatase inhibitor letrozole (PHR1540, Sigma) or β-estradiol (E2758, Sigma) was administered to eggs incubating at 32°Cand 26°C. Letrozoleand β-estradiol was dissolved in 95% ethanol at a concentration of 10 μg/μl, and 10 μl of the drug was applied topically to the eggshell in the region adjacent to the embryo at stage 16. Controls were treated with 10 μl of 95% ethanol. Gonads were separated from the adjacent mesonephros at stage 17, 18, 19 and 20, and preserved in TRIzol (Invitrogen) for qRT-PCR analysis.

Preparation of lentivector-*Kdm6b*-shRNA constructs

Three shRNAs targeting turtle *Kdm6b* mRNA were designed to give rise to siRNA. The lentivirus vector was used to deliver shRNAs directed specifically against turtle *Kdm6b* mRNA. Each designed shRNA construct contained a unique 21 nt double-stranded *Kdm6b* sequence that presented as an inverted complementary repeat, a loop sequence (5'-CTCGAG-3') and the RNA Pol-II terminator (5'-TTTTTT-3'). Annealed oligonucleotides were ligated into pGP-U6 (GenePhrama) between the *Bbs* and *Xho* sites by T4 DNA ligase (TaKaRa) to produce pGP-U6-Kdm6b-shRNA. The pGP-U6- Kdm6b-shRNA construct was digested with *Age*I-*Eco*RI and inserted into the *Eco*RI site of pGLV-U6-GFP (GenePharma, Shanghai, China). The recombinant vector pGLV-GFP-Kdm6b-shRNAwas termed LV-Kdm6b-shRNA. The negative control vector (pGLV-GFP-NC-shRNA, termed LV-NC-shRNA) contained a nonsense shRNA insert in order to control any effects caused by non-RNAi mechanisms. The sequences of the shRNAs are as follows: *Kdm6b*-shRNA#1, 5'-GAAGTTCAAGGAGTCGTATCT-3';*Kdm6b*-shRNA#2,5'-GGTGCAGCTCTACATGAAA GT-3'; *Kdm6b*-shRNA#3, 5'- GCCACCAGGAGAACAACAACT -3'; negative control, 5'-TTCTCCGAACGTGTCACGT-3'.

For the generation of lentivirus, 293 T producer cells were transfected with optimized packaging plasmids (pGag/Pol, pRev and pVSV-G) along with pGLV-Kdm6b-shRNA or pGLV-NC-shRNA expression clone construct by lipofectamine. 24 h post transfection, the transfection mix was replaced by a fresh culture medium (without antibiotics). The virus-containing supernatant was harvested 72 h post transfection, cleared by centrifugation (3000 rpm/min, 15 min, and 4°C), and then filtered through a 0.45 μm filter (Millipore). Viruses were titrated by adding serial dilutions to fresh 293 T, and assessing GFP expression

after 48 h. Viral titres of approximately 5×10^8 infectious units/ml were obtained. Lentivirus aliquots were stored at -80 °C before infection of turtle embryos.

Preparation of lentivector-*Dmrt1* over-expression construct

Total RNA was isolated from MPT embryonic gonads of stage 25, where upon reverse transcription was carried out to prepare cDNA. A full-length turtle *Dmrt1* open reading frame (1107bp) was PCR amplified from cDNA using forward primer 5'-CCCCAAATTGTAGAG GCGAACC-3' and reverse primer 5'-TGAGGGCAGGGCAGAGGAGG-3'. The PCR product was digested with *EcoR*I and cloned to pGLV-EF1a-GFP (LV-4,GenePharma, Shanghai, China). The recombinant vector pGLV-GFP-Dmrt1 was named LV-Dmrt1. High quality proviral DNA was used to transfect 293 T cells. Virus propagation was carried out as described above. A viral titre of 5×10^8 infectious units/ml was obtained.

Infection of turtle embryos

High titre virus of LV-*Kdm6b*-shRNA (5×10^8 Infectious units/ml) was injected into stage 13 turtle embryos at 26°C, or combination with LV-*Dmrt1*-OE virus. Eggs were swabbed with alcohol swabs prior to injection using a fine (0-25μl) metal Hamilton needle. Approximately 5 μl was injected per embryo, and 300-500 eggs were injected in each treatment group. 100 control embryos at MPT and FPT were injected with scrambled control virus of LV-NC-shRNA. Eggs were sealed with parafilm and incubated for the indicated time points (stage 14-25). Ratio of survival to stage 21 was 20-50%. Embryos showing GFP fluorescence in the urogenital system were chosen for further analysis. One of each pair of GFP positive gonads was dissected for qRT-PCR, and the other gonad was preserved for histological and immunofluorescence analysis. The GFP^+ gonads showing >70% reduction of *Kdm6b* expression were analyzed in knockdown experiment, and those GFP^+ gonads showing >70% downregulation of *Kdm6b* and >20-fold upregulation of *Dmrt1* were chosen for rescue experiment.

Western blot

Twenty pairs of stage 21 gonads from each group were pooled together and extracted with RIPA lysis buffer (Santa Cruz, USA) . Equal amounts of denatured protein samples were separated on 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was incubated in 5% dry skim milk at room temperature for 1 h and subsequently with primary antibody (rabbit anti H3K27me3, Millipore, 07-449) at dilution 1:1000 overnight at 4°C. Antibody recognition was detected with the secondary antibody linked to horseradish peroxidase (goat anti-rabbit IgG-HRP, 1:5000, Santa Cruz, USA) at room temperature for 60 min. H3 bands (rabbit polyclonal pan-H3, Abcam, ab1791, 1:3000) were used as an internal control. The immunoreactive bands were visualized with Maxi ECL kit (MultiSciences, Hangzhou, China).

ChIP analysis

Twenty-five pairs of stage 15 turtle gonads were pooled into a single tube for each group at stage 15. Protein and DNA were cross-linked with 1% formaldehyde before cell lysis. Gonadal cells were lysed and sonicated according to previously described methods (23).

Immunoprecipitation was performed using a ChIP assay kit (Upstate) according to the manufacturer's protocol. Sonicated lysates were incubated overnight at 4°C with rabbit anti-KDM6B (produced privately through Sangon Biotech), rabbit polyclonal H3K27me3(Millipore, 07-449), rabbit polyclonal pan-H3 (Abcam, ab1791), or IgG as a negative control. After denaturation of cross-links, immunoprecipitated DNA was purified using the Qiagen PCR Purification kit (Qiagen, Valencia, CA). The purified DNA was quantified by real-time PCR with Maxima SYBR Green qPCR Master Mix. The comparative threshold cycle (Ct) method was used for relative quantification. The sequences of primers for ChIP-qPCR are listed in Table S2.

Statistical analyses

Each experiment was independently repeated at least 3 times. All data was expressed as the means ± S.D. The student's unpaired *t*-test was used to test significance (*, $^{\#}$, $P< 0.05$; **, $^{\#\#}$ $P< 0.01$; ***, $^{\#\#\#}$ $P< 0.001$; n.s., no significance).

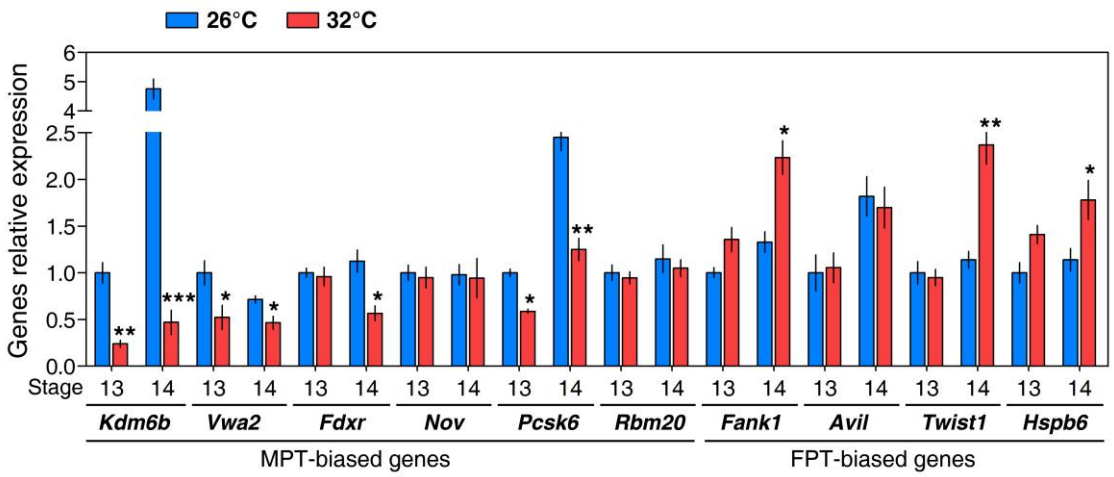

Fig. S1. mRNA expression of male- and female-producing temperature-biased genes in gonad-mesonephros complexes of stage 13 and 14.

Expression levels were determined by qRT-PCR, and were normalized to *Gapdh*. The expression level in 26°C gonad-mesonephros complexes of stage 13 was defined as 1 for each gene. MPT: male-producing temperature; FPT: female-producing temperature. Data are means ± SD.*P< 0.05; **P< 0.01; ***P< 0.001; n=3.

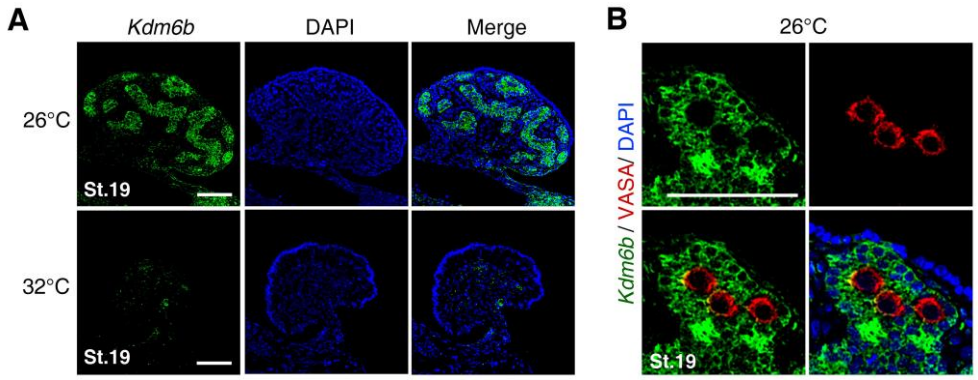

Fig. S2. mRNA localization of *Kdm6b* in gonadal sections of stage 19 embryos at 26°C and 32°C.

(**A**)Fluorescence in situ hybridization with an antisense probe specific to *Kdm6b*. Scale bar,50 µm. (**B**) Enlarged image of 26°C embryonic gonadal section (stage 19) double-stained for VASA protein (red; germ cells); DAPT (blue); and *Kdm6b* transcripts (green). Scale bar, 50 µm.

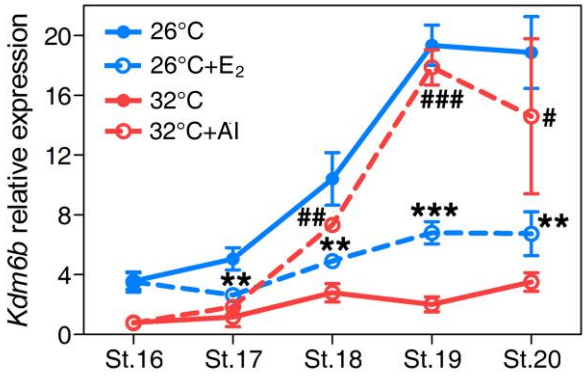

Fig. S3. Timecourse response of *Kdm6b* expression to hormone treatment in vivo.
Stage 16 eggs were treated with β-estradiol (E_2) or letrozole (AI) at stage 16, and gonads were dissected at different stages for qRT-PCR analysis. The expression level in 32°C gonads of stage 16 was defined as 1, following normalization to *Gapdh*. Data are means ± SD; $*^{,\#}P<$ 0.05; $**^{,\#\#}P<$ 0.01; $***^{,\#\#\#}$ $P<$ 0.001; n=3.

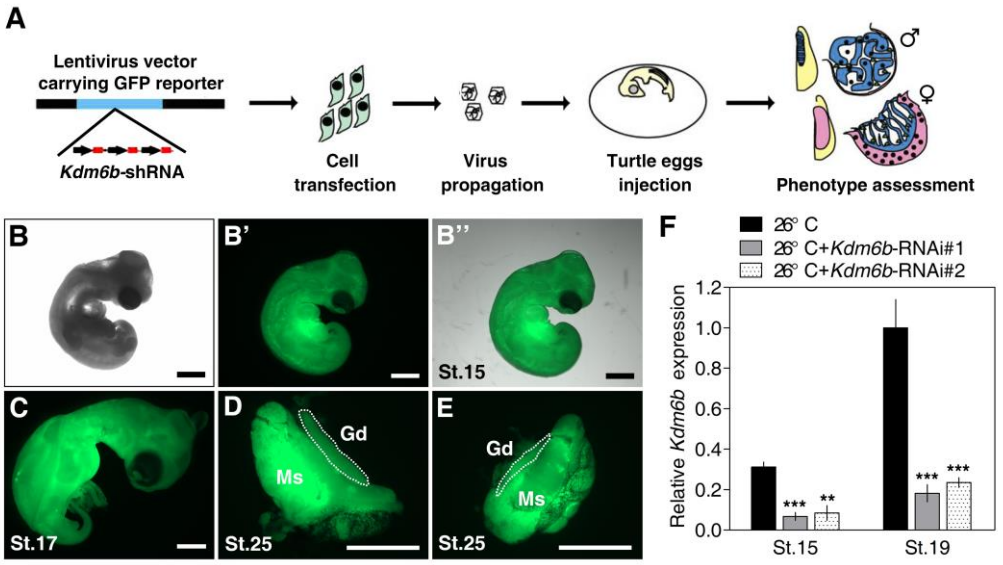

Fig. S4. Establishment of *Kdm6b*-knockdown turtle model using lentiviral infection. (**A**) Experimental design for *Kdm6b* knockdown in turtle embryos.(**B**) Bright and epifluorescence images of whole embryos of stage 15 infected with scrambled lentiviral vector (LV-NC) at stage 13. (**C**) Epifluorescence image of embryos at stage 17 with virus injection. Scale bars are 1 mm. (**D**, **E**) Robust GFP expression in gonad-mesonephros complexes from stage 25 embryos treated with LV-*Kdm6b*-shRNA#1. Gd, gonad; Ms, mesonephros. Scale bars are 1 mm. (**F**) *Kdm6b* expression in 26°C gonads following treatment of LV-*Kdm6b*-shRNAs. 26°C eggs were injected with lentivirus carrying *Kdm6b*-shRNAs at stage 13, and GFP positive gonads were dissected at stage 15 and 19 for qRT-PCR analysis. The expression level in 26°C gonads of stage 19 was set as 1, after normalization to *Gapdh*. Data are means ± SD; ***$P < 0.001$; n=3.

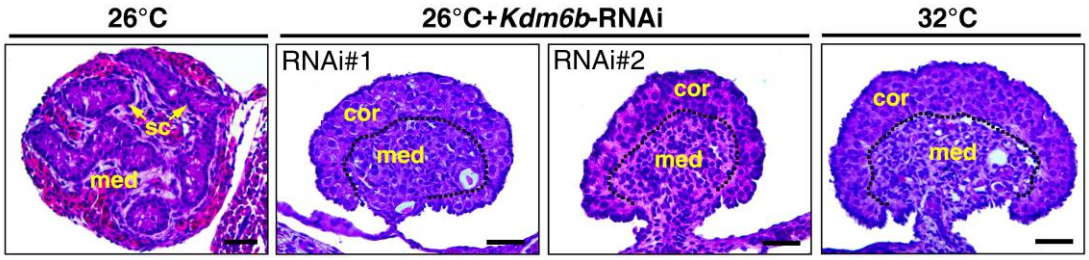

Fig. S5. Histological analysis of *Kdm6b*-deficient 26°C gonads at stage 25.
H&E staining of gonadal sections from 26°C, 26°C+*Kdm6b*-RNAi#1,26°C+*Kdm6b*-RNAi#2 and 32°C embryos at stage 25. The dashed black line indicates the border between medulla and cortex.sc, seminiferous cord; cor, cortex; med, medulla; scale bars are 50 μm.

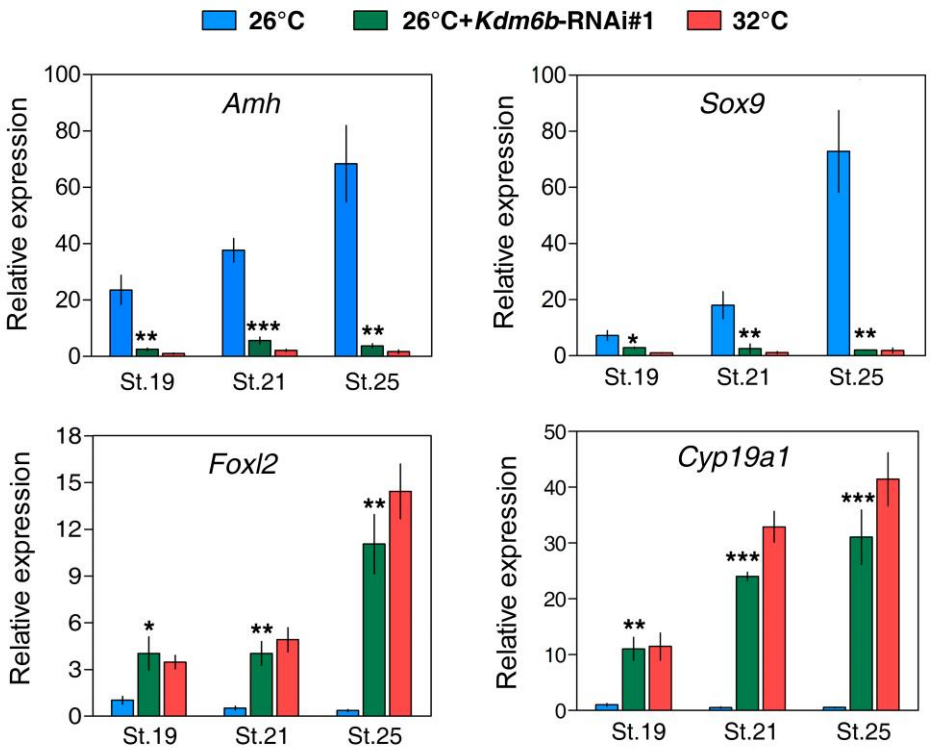

Fig. S6. Responses of male- and female-specific markers to *Kdm6b* knockdown in 26°C gonads.

qRT-PCR of *Amh*, *Sox9*, *Foxl2* and *Cyp19a1* in gonads from 26°C, 26°C+*Kdm6b*-RNAi#1 and 32°C embryos at stage 19, 21 and 25. The expression level in 32°C gonads of stage 19 was defined as 1 for *Amh* and *Sox9* analysis, and the expression level of 26°C at stage 19 was set as 1 for *Foxl2* and *Cyp19a1*. Data are means ± SD; *, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$; n=3.

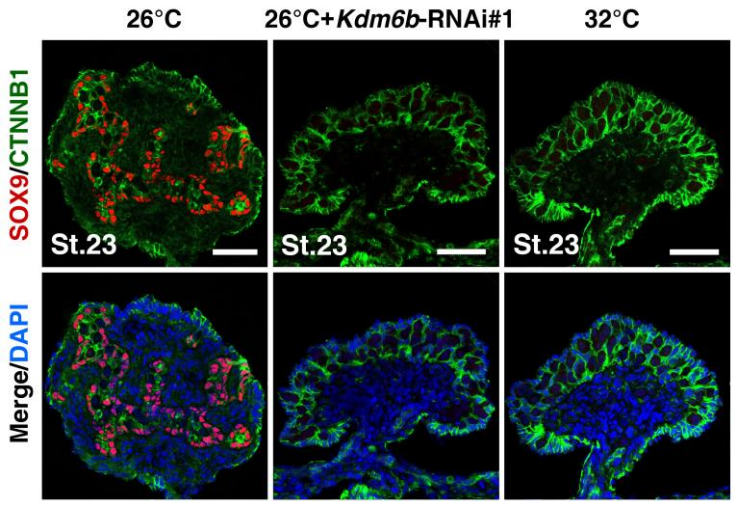

Fig. S7. Response of SOX9 protein expression to *Kdm6b* knockdown in 26°C gonads of stage 23.

Immunofluorescence of SOX9 and CTNNB1 (β-catenin) in gonadal sections from indicated embryos at stage 23. Scale bars are 50 μm.

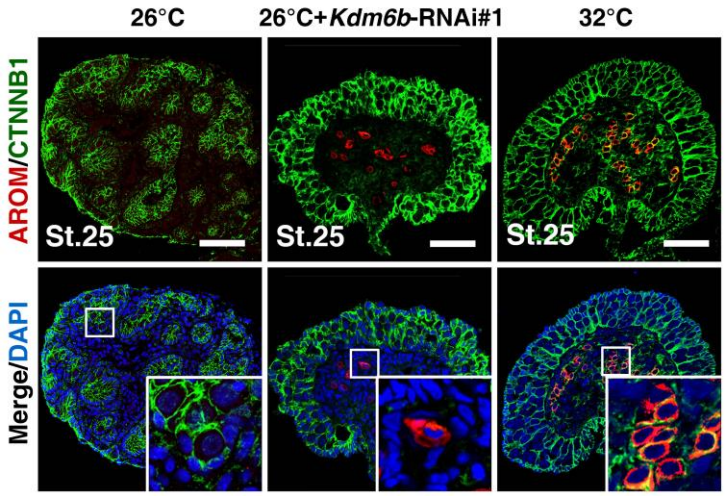

Fig. S8. Aromatase expression in 26°C gonads with *Kdm6b* knockdown at stage 25. Immunofluorescence of aromatase and CTNNB1 (β-catenin) in gonadal sections from indicated embryos at stage 25. AROM, aromatase. Scale bars are 50 μm.

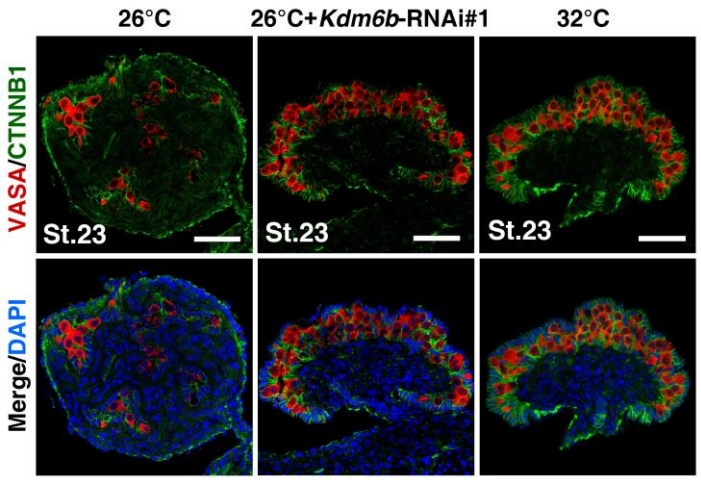

Fig. S9. Germ cell distribution in 26°C gonads of stage 23 following *Kdm6b* knockdown. Immunofluorescence of VASA and CTNNB1 (β-catenin) in gonadal sections from indicated embryos at stage 23. Scale bars are 50 μm.

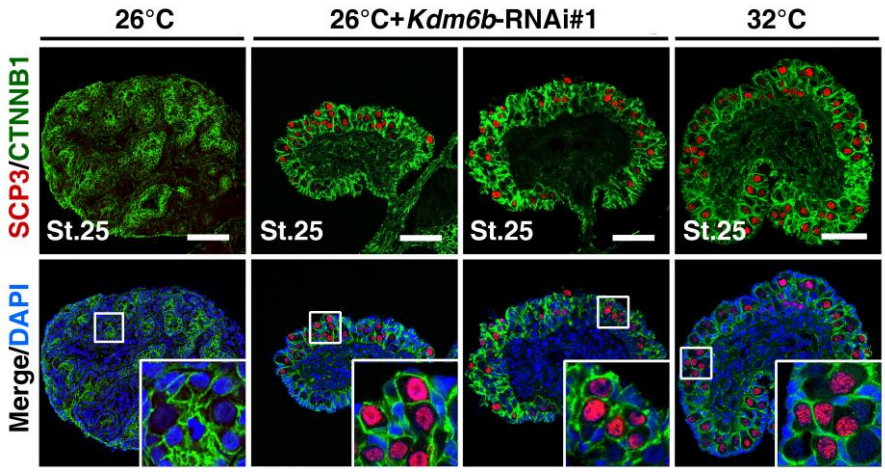

Fig. S10. Meiotic status of germ cells in26°C gonads with *Kdm6b* knockdown at stage 25. Immunofluorescence of SCP3 (meiotic marker) and CTNNB1 (β-catenin) in gonadal sections from indicated embryos at stage 25. Scale bars are 50 μm.

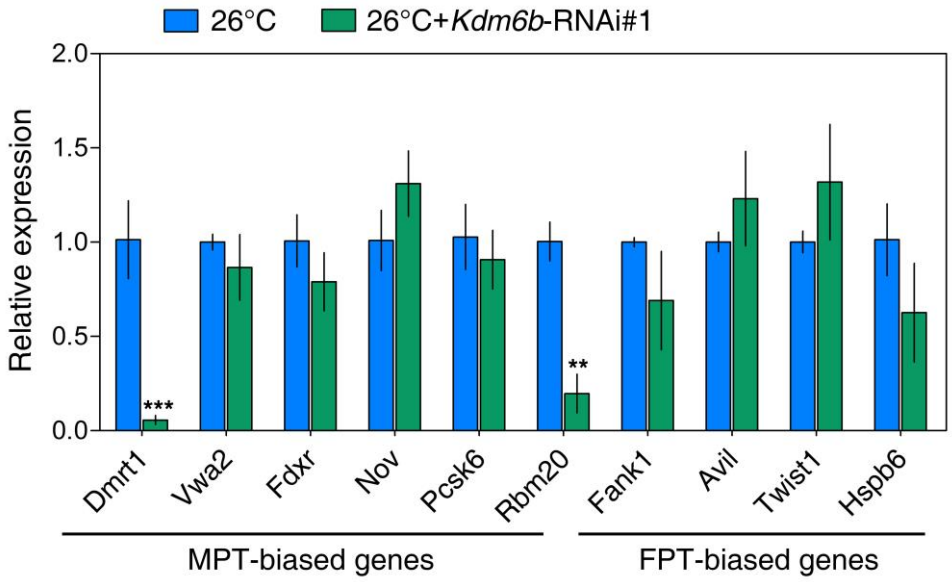

Fig. S11. Responses of MPT- and FPT-biased genes to knockdown of *Kdm6b* at stage 15. Expression levels of MPT- and FPT-biased genes in 26°C gonads following *Kdm6b* knockdown were determined by qRT-PCR, and were normalized to *Gapdh*. The expression level in control 26°Cgonads was defined as 1 for each gene. MPT: male-producing temperature; FPT: female-producing temperature. Data are means ± SD. ***P*< 0.01; ****P*< 0.001; n=3.

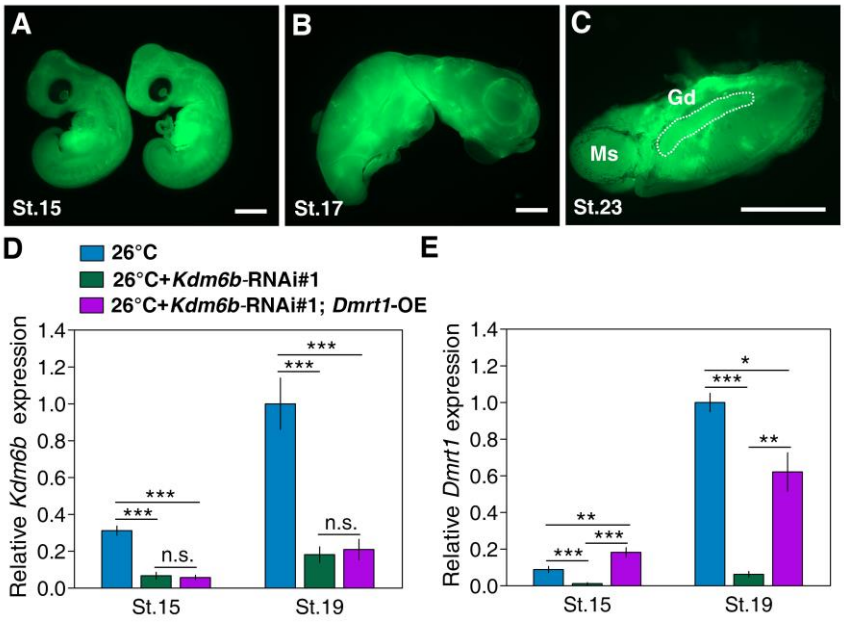

Fig. S12. Establishment of *Kdm6b*-knockdown and *Dmrt1* overexpression turtle model. (**A, B**) Epifluorescence image of whole embryos of stage 15(**A**) and stage 17(**B**) infected with lentiviral vectors LV-*Kdm6b*-shRNA#1 and LV-*Dmrt1*-OE at stage 13. (**C**) GFP expression in gonad-mesonephros complexes from stage 2326°Cembryos treated with LV-*Kdm6b*-shRNA#1 and LV-*Dmrt1*-OE. Gd, gonad; Ms, mesonephros. Scale bars are 1 mm. (**D, E**) mRNA expression of *Kdm6b* (**D**) and *Dmrt1*(**E**) in 26°C gonads following treatment of LV-*Kdm6b*-shRNA#1 and LV-*Dmrt1*-OE. 26°C eggs were injected with two lentiviral vectors at stage 13, and pairs of GFP-positive gonads were dissected at stage 15 and 19 for qRT-PCR analysis. The expression level in 26°C gonads of stage 19 was set as 1, after normalization to *Gapdh*. Data are means ± SD; **P*<0.05;***P*< 0.01;****P*< 0.001;n.s., no significance; n=3.

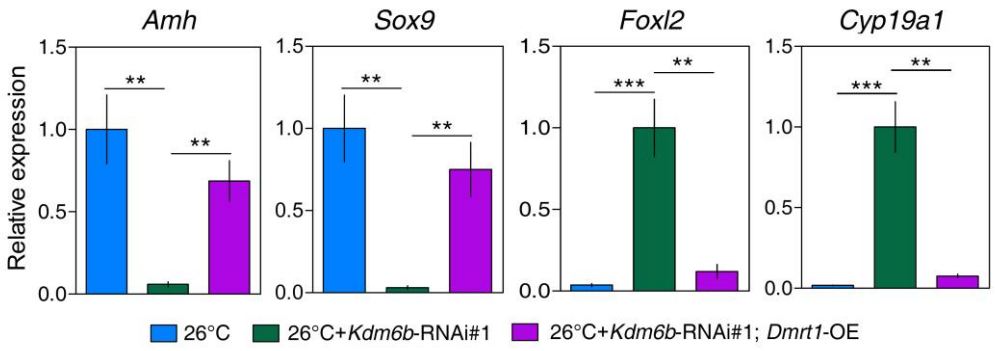

Fig. S13. The mRNA expression of sex-specific markers in stage 25 *Kdm6b*-knockdown 26°C gonads following *Dmrt1* overexpression.

qRT-PCR of *Amh*, *Sox9*, *Foxl2* and *Cyp19a1* in gonads from indicated embryos at stage 25.The highest expression level was defined as 1 for each gene, after normalization to *Gapdh*. Data are means ± SD. ***P*< 0.01; ****P*< 0.001; n=3.

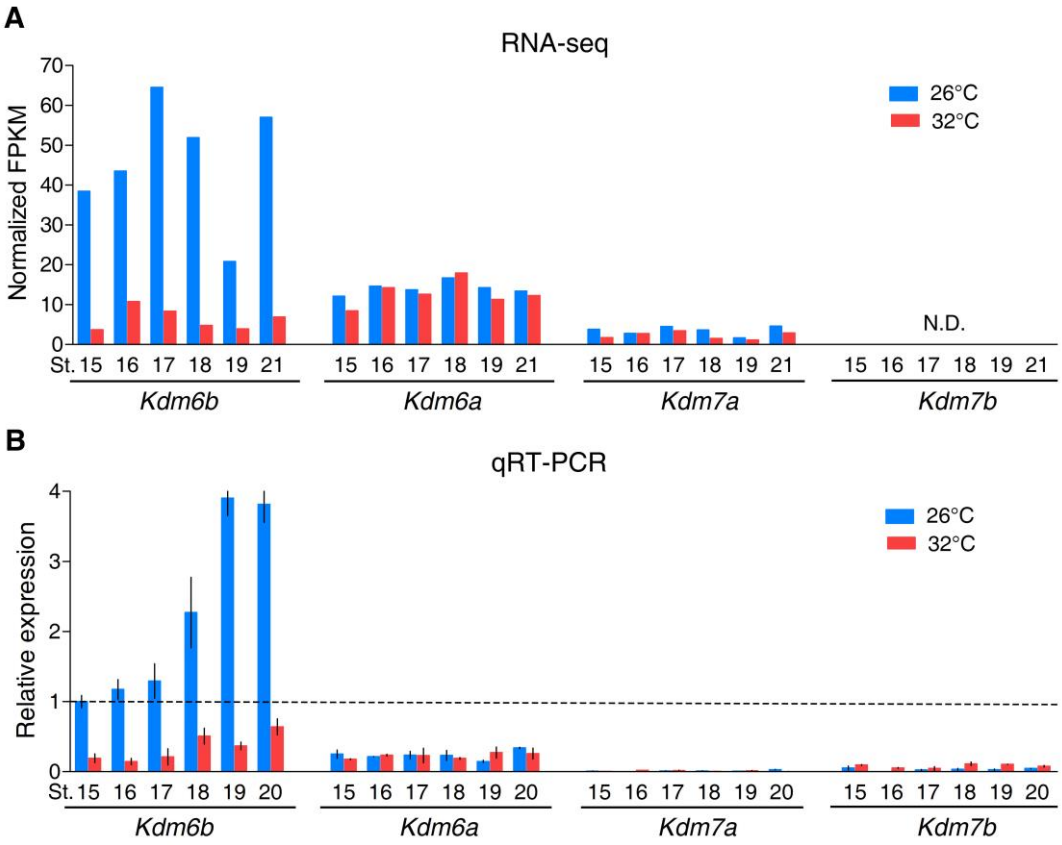

Fig. S14. *Kdm6b* is the most highly expressed enzyme that demethylates H3K27 in stage 15-21 turtle gonads at 26°C or 32°C.

(**A**) RNA-seq measurement showing the transcript expression levels for the 4 members of the H3K27 demethylases family(*Kdm6a*, *Kdm6b*, *Kdm7a* and *Kdm7b*) in early embryonic gonads of both temperatures. All expression columns are shown with normalized FPKM values. (**B**) Validation of mRNA expression levels of *Kdm6a*, *Kdm6b*, *Kdm7a* and *Kdm7b* by qRT-PCR analysis. The expression level of *Kdm6b* in 26°C gonads of stage 15 was defined as 1, after normalization to *Gapdh*. Data are means ± SD. n=3.

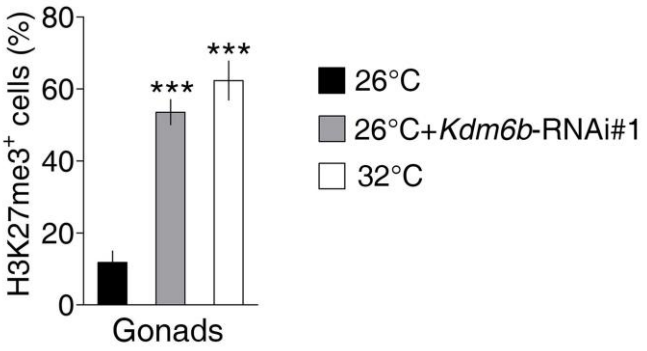

Fig. S15. The ratio of H3K27me3 positive cells to total gonadal cells (stained with DAPI) in stage 21 gonads.

Data are means ± SD. ****P*< 0.001; n=8.

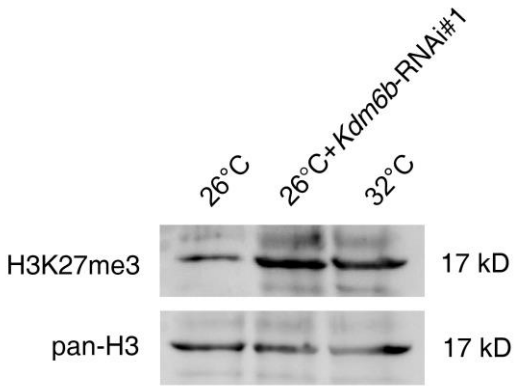

Fig. S16. The protein expression of H3K27me3 in 26°C gonads with *Kdm6b* knockdown was similar to FPT levels.

Western blot analysis of the total H3K27me3 level of indicated gonads at stage 21.

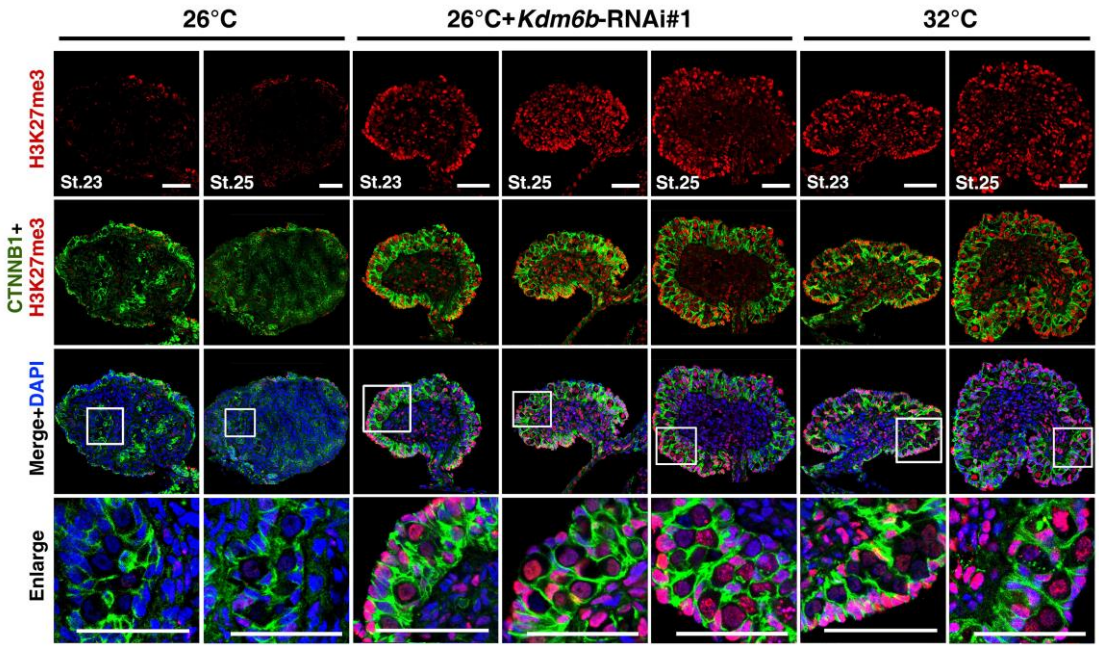

Fig. S17. The methylation status of histone H3K27 in 26°C gonads with *Kdm6b* knockdown.

Immunofluorescence of H3K27me3 and CTNNB1 in gonadal sections of 26°C, 26°C +*Kdm6b*-RNAi#1, and 32°C embryos at stage 23 and 25.Scale bars are 50 μm.

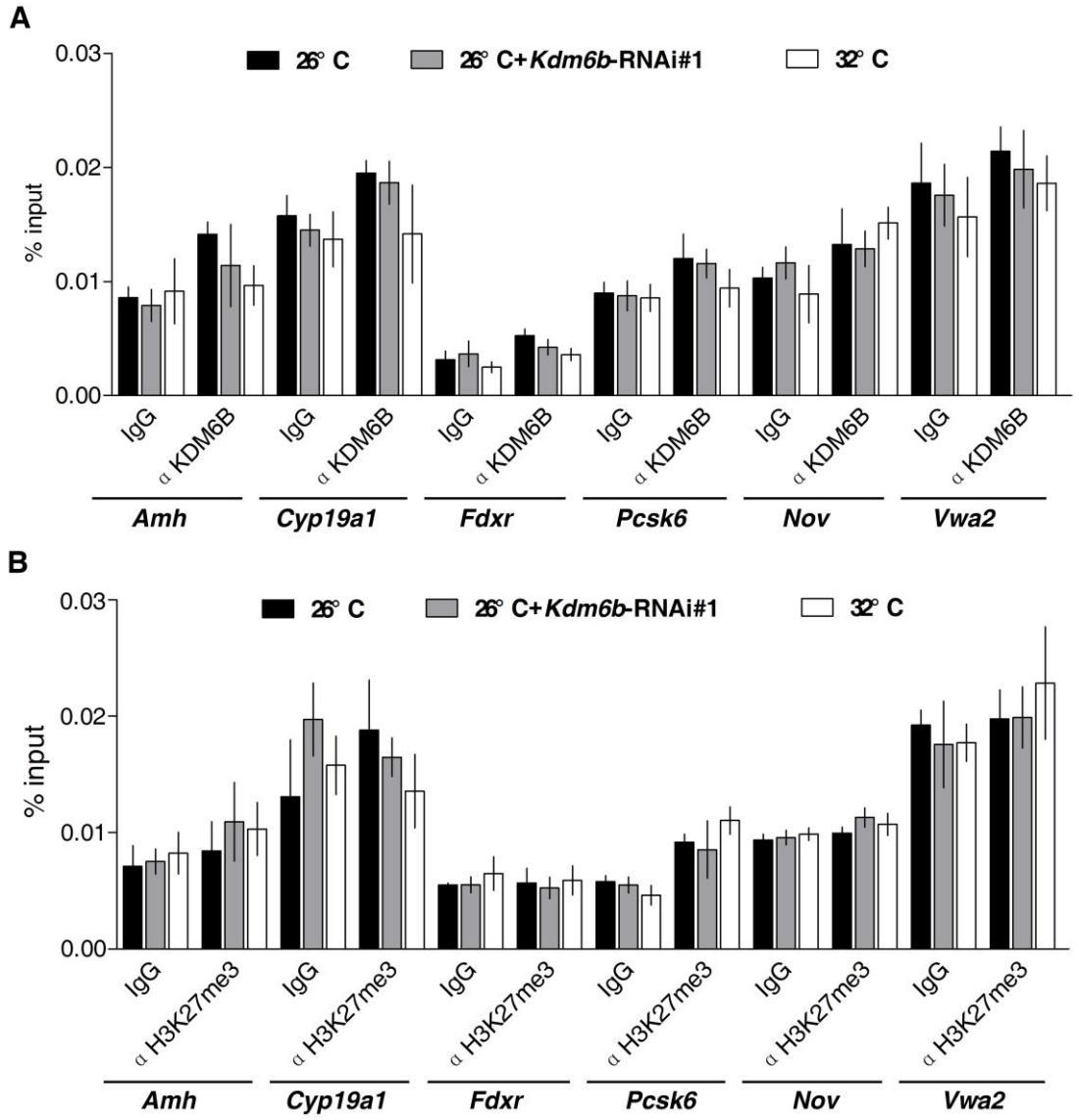

Fig. S18. The levels of KDM6B and H3K27me3 at *Amh*, *Cyp19a1*, *Fdxr*, *Pcsk6*, *Nov* and *Vwa2* in 26°C gonads were not significantly altered following *Kdm6b* knockdown. ChIP-qPCR assays with antibodies specific for KDM6B (**A**) and H3K27me3 (**B**) at the indicated loci in 26°C, 26°C+*Kdm6b*-RNAi#1, and 32°C gonads of stage 16. Signals were shown as a percentage of the input. Data are means ± SD. **P*< 0.05, ***P*< 0.01; n=3.

Table S1. The proportion of *Kdm6b*-deficient 26°C gonads developing as ovaries following overexpression of *Dmrt1*.

Incubation temperature	Viral treatment	No. of injected eggs	No. of embryos survival to St.21	No. of GFP^+ embryos	No. of testes	No. of ovaries	Sex ratio (% ovaries)
26°C	*Kdm6b*-RNAi#1	318	74	45	6	39	**39/45(86.7%)**
26°C	*Kdm6b*-RNAi#1; *Dmrt1*-OE	500	61	18	16	2	**2/18(11.1%)**

Phenotype of gonads was assessed by histology, SOX9 immunostain and qRT-PCR.

Table S2. Primer list

Experiments	Direction of Primer	Primer sequence (5'-3')
qRT-PCR		
Kdm6b	F	ACGTGAAATCCATTGTGCCCAT
	R	AGGCGATCTTCTTCCCCGAAC
Dmrt1	F	ACTACCCTCCTGCCTCCTACCT
	R	CTCCTTTGGTGCTTTCATTGCT
Amh	F	CGGCTACTCCTCCCACACG
	R	CCTGGCTGGAGTATTTGACGG
Sox9	F	CAGTCCGAGCCATTACAGCG
	R	GCGGGTGATGGTCGGGTA
Foxl2	F	ACGAGTGCTTCATCAAGGTGCCC
	R	TCCGCCGCCGTCGGTAGTT
Cyp19a1	F	GCACATGGACTTTGCATCACA
	R	GAACCATCATCTCCAACACACACTGGTTC
Vwa2	F	CTAACCAAGCAGGAAGTAAAGGAG
	R	CAGGATTTCAGGGACAGAGGA
Fdxr	F	GGGGTTTATTCAGCAAGAGCG
	R	CAGAACTCGGGCAACATCCA
Pcsk6	F	TCCAGGGATAACTGCGTCAAG
	R	GGGCTGCGTTCAGGAATAGG
Now	F	TTCAAAGGAAGGTCGGTATTCT
	R	TCCTGGCTTGAGCACTAGATG
Rbm20	F	AACTCTACAACCCTGAAGAACCG
	R	TTCGGGAACTACTGAAACTTGG
Hspb6	F	CCCTTTGGGACAGCAGAATT
	R	CCAGCAGGTGAGTGGAGTTTAT
Avil	F	GTTCGCCTCGTCGCTCAAC
	R	CGCCGTCACAGATGGTTGC
Twist1	F	GATAGGCATCTGCACAATCCA
	R	AACGGCGAAAGTCCACAATA
Fank1	F	GGCTTTACTGCTCTGATGGTTG
	R	CATTAGGCTGTCCTTTCCACTT
Gapdh	F	GGCTTTCCGTGTTCCAACTC
	R	GACAACCTGGTCCTCCGTGTATC
ChIP-qPCR		
Dmrt1 promoter	F	TGGCAGCCCTATGGCATTT
	R	GGTAGCAGGTCCCTTGGTG
Amh first exon	F	CCCTCAACTGCTCTGCTA
	R	CTCCAAATCCCAACTCCT
Cyp19a1 promoter	F	ATAACAGTATCTGCCTCCG
	R	GGTTCCTTGCTGGTCCCT
Fdxr first exon	F	CTGGAAGTGGCGGTCTCC

	R	GGTCCACTCCCCACAACA
Pcsk6 first exon	F	GGCTTGGCTGCTGCTCCTC
	R	CCTGCACTGCCCAGTGGTTG
Nov promoter	F	GCCACGGGCTGGAAGTTAG
	R	GCGGCGAGGGAACCAAT
Vwa2 promoter	F	GCAGGCAAGCGAAACTGA
	R	CGTCCCGACAACTTTACCACT

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