SEX DETERMINATION

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

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Temperature-dependent sex determination is a notable model of phenotypic plasticity. In many reptiles, including the red-eared slider turtle *Trachemys scripta elegans* (*T. scripta*), the individual's sex is determined by the ambient temperature during egg incubation. In this study, we show that the histone H3 lysine 27 (H3K27) demethylase KDM6B exhibits temperature-dependent sexually dimorphic expression in early *T. scripta* embryos before the gonad is distinct. Knockdown of *Kdm6b* at 26°C (a temperature at which all offspring develop into males) triggers male-to-female sex reversal in >80% of surviving embryos. KDM6B directly promotes the transcription of the male sex-determining gene *Dmrt1* by eliminating the trimethylation of H3K27 near its promoter. Additionally, overexpression of *Dmrt1* is sufficient to rescue the sex reversal induced by disruption of *Kdm6b*. This study establishes causality and a direct genetic link between epigenetic mechanisms and temperature-dependent sex determination in a turtle species.

In many reptiles, including the red-eared slider turtle *Trachemys scripta elegans* (*T. scripta*), gonadal sex is determined by the environmental temperature experienced during embryogenesis (*1*–*4*). However, the molecular mechanisms underlying this phenotypic plasticity have remained elusive. Recently, epigenetic marks, such as DNA methylation and histone modifications of known regulators of gonadal differentiation, have been shown to differ between temperatures in species with temperature-dependent sex determination (*5*–*11*). However, all available reports are correlative, and whether the differential epigenetic status is a cause or consequence of sexual development in species for which sex is determined by temperature has not been elucidated. Here we provide molecular and genetic evidence that the epigenetic regulator *Kdm6b* plays a causal role in male sex determination by demethylating H3K27me3 (trimethylated histone H3 lysine 27) at the promoter of *Dmrt1*.

Trimethylation of H3K27 contributes to transcriptional repression in many organisms (*12*). KDM6B (also called JMJD3) is a histone demethylase that specifically demethylates H3K27me3 and is involved in transcriptional activation during normal development (*13*–*16*). We previously sequenced the *T. scripta* gonadal transcriptome during developmental stages 15 to 21 at male-producing (26°C) and female-producing (32°C) temperatures and found that *Kdm6b* was up-regulated at 26°C (*17*). A reverse transcription quantitative real-time fluorescence polymerase chain reaction (qRT-PCR) analysis revealed that the 26°C-specific expression of *Kdm6b* began in *T. scripta* gonad-mesonephros complexes as early as stage 13, before the gonad was distinct (Fig. 1A and fig. S1). This sexually dimorphic expression profile was maintained in gonads throughout the temperature-sensitive period (stages 15 to 20) (Fig. 1B). Immunofluorescence and in situ hybridization showed that mRNA and protein of *Kdm6b* were detected in gonadal somatic cells of seminiferous cords but not germ cells (Fig. 1C and fig. S2), implying that KDM6B functions in somatic cells to regulate the sexual development of *T. scripta*. We next examined the responses of *Kdm6b* expression to temperature shifts and sex hormone–induced sex reversal during the temperature-sensitive window. In gonads shifted from either 26° to 32°C or 32° to 26°C at stage 16, significant changes in *Kdm6b* expression were evident by stage 17, preceding gonadal sex differentiation (Fig. 1D). In addition, *Kdm6b* responded quickly to estrogen treatment at 26°C by stage 17 and to treatment at 32°C with the aromatase inhibitor by stage 18 (fig. S3). These expression profiles suggest that *Kdm6b* is an early responder to temperature or hormone treatments, with the potential to act as a master regulator of somatic gene expression at 26°C.

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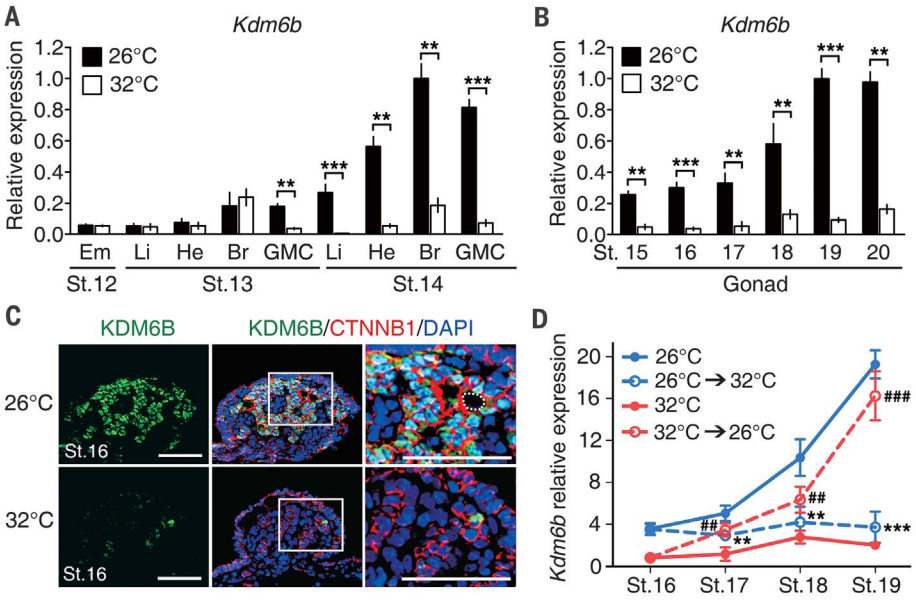
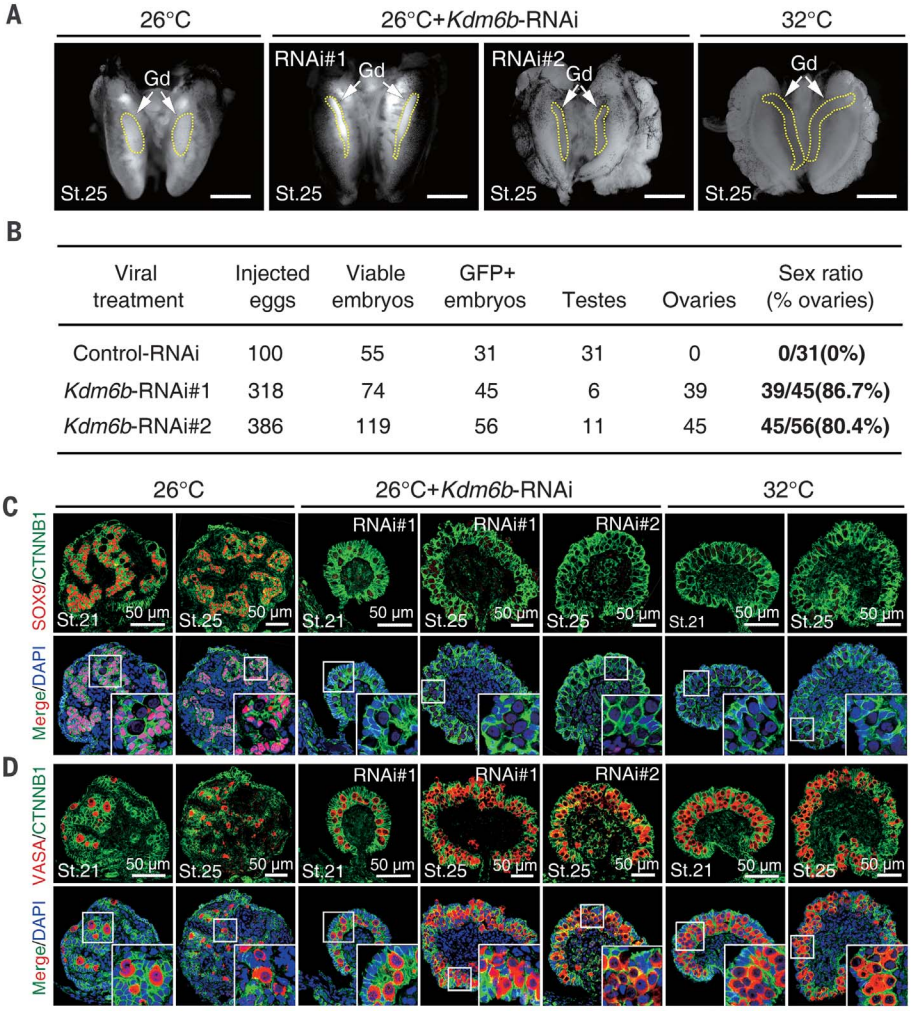


Fig. 1. *Kdm6b* exhibits a temperature-dependent sexually dimorphic expression pattern in early gonads of *T. scripta*. (**A** and **B**) Results from qRT-PCR analysis of *Kdm6b* in (A) whole embryos at stage 12 and different embryonic tissues at stages 13 and 14 and (B) embryonic gonads at stages 15 to 20. Analyses were conducted at both 26° and 32°C. Expression was normalized to *Gapdh*. The relative expression levels, both measured at 26°C, in the brain at stage 14 and the gonad at stage 19 were defined as 1.0 in (A) and (B), respectively. St, stage; Em, embryo; Li, liver; He, heart; Br, Brain; GMC, gonad-mesonephros complex. (**C**) Coimmunofluorescence of KDM6B (green) and CTNNB1 (β-catenin, red) in gonadal sections of stage 16 embryos at 26° and 32°C. The panels at right are higher-magnification views of the boxed areas in the middle panels. The dotted circle outlines a germ cell. DAPI, 4′,6-diamidino-2-phenylindole. Scale bars, 50 μm. (**D**) Time course response of *Kdm6b* expression to temperature shifts in vivo at each stage from 16 to 19. Gonads were dissected for qRT-PCR analysis. Results were normalized to *Gapdh*, and the expression level in stage 16 gonads at female-producing temperature (32°C) was defined as 1. Data in (A), (B), and (D) are means ± SD, n = 3 biological replicates. ** $^{\#\#}P < 0.01$; *** $^{\#\#\#}P < 0.001$.

We previously established a method of introducing short hairpin RNAs (shRNAs) in ovo during early stages of *T. scripta* embryonic development that results in 30 to 50% viability (*18*). To investigate the functional role of *Kdm6b* in sex determination of *T. scripta*, we used RNA interference (RNAi) to generate loss-of-function mutants by injecting lentivirus carrying an shRNA specific to *Kdm6b* into 26°C embryos at stage 13 (fig. S4). Approximately 20 to 50% of injected embryos survived to stage 21. Lentiviral treatment of two different shRNAs led to a 73 to 82% reduction of *Kdm6b* transcripts in 26°C gonads from early stage 15 onward (fig. S4), as compared with treatment with nonsilencing scrambled virus. Control 26°C embryos treated with the scrambled virus exhibited typical cylindrically shaped testes, and control 32°C embryos displayed typical long and flat ovaries (Fig. 2A). *Kdm6b*-deficient 26°C gonads became elongated and exhibited varying degrees of female-like morphology (Fig. 2A), characterized by a thickened outer cortex containing a number of primordial germ cells and degenerated medullary cords (fig. S5). Overall, two independent experiments with different shRNAs showed that 39 of 45 (86.7%) and 45 of 56 (80.4%) *Kdm6b*-knockdown embryos displayed a complete male-to-female shift in sexual trajectory at 26°C (Fig. 2B).

To confirm the activation of the female pathway in 26°C embryos with *Kdm6b* knocked down, we analyzed the expression of the testicular Sertoli cell markers *Amh* and *Sox9* and the ovarian regulators *Cyp19a1* and *Foxl2* in gonads after sex determination at stages 19, 21, and 25. qRT-PCR analysis showed that expression of *Amh* and *Sox9* sharply decreased, whereas expression of *Cyp19a1* and *Foxl2* significantly increased in *Kdm6b*-deficient 26°C gonads relative to controls (fig. S6). SOX9 protein was expressed specifically in the nuclei of precursor Sertoli cells in control 26°C gonads, whereas it was sharply reduced or absent in *Kdm6b*-deficient 26°C gonads (Fig. 2C and fig. S7), where ectopic activation of aromatase was detected in the gonadal medulla (fig. S8). Immunofluorescence showed that VASA-positive germ cells, some of which were labeled with the meiotic marker SCP3, exhibited a female-like distribution pattern in the developed outer cortex of *Kdm6b*-knockdown 26°C gonads (Fig. 2D and figs. S9 and S10). These data provide functional evidence that disruption of *Kdm6b* leads to female development at 26°C, indicating that high transcript levels of *Kdm6b* are critical to activate the male pathway in this temperature-dependent sex determination system.

To address the molecular basis of this sex-reversal phenotype induced by knockdown of *Kdm6b*, we aimed to identify the target genes responsible for regulation of temperature-dependent sex determination by *Kdm6b*. Of the six earliest male-biased genes previously reported (*17*), only *Dmrt1* and *Rbm20* displayed >50% reduction of mRNA expression in response to *Kdm6b* knockdown at stage 15 (fig. S11). *Dmrt1* was of particular interest because the early male-specific expression pattern is detected at stage 14 (*18*), just after dimorphic expression of *Kdm6b* is detected at stage 13 (Fig. 1A). In addition, we previously demonstrated that the loss of *Dmrt1* redirected gonads incubating at 26°C toward female fate, whereas the gain of *Dmrt1* redirected gonads incubating at 32°C toward male fate (*18*). *Dmrt1* mRNA levels were reduced to ~13% in *Kdm6b*-deficient 26°C gonads from stage 15 onward (Fig. 3A), and DMRT1 protein was also reduced or absent (Fig. 3B). This observation indicates that *Dmrt1*



Viral treatment	Injected eggs	Viable embryos	GFP+ embryos	Testes	Ovaries	Sex ratio (% ovaries)
Control-RNAi	100	55	31	31	0	**0/31(0%)**
Kdm6b-RNAi#1	318	74	45	6	39	**39/45(86.7%)**
Kdm6b-RNAi#2	386	119	56	11	45	**45/56(80.4%)**

Fig. 2. Knockdown of *Kdm6b* at 26°C leads to male-to-female sex reversal in *T. scripta*. (**A**) Representative images of the gonad-mesonephros complexes from 26°C control, 26°C loss-of-function mutants (*Kdm6b*-RNAi#1 and *Kdm6b*-RNAi#2), and 32°C control embryos at stage 25. Gd, gonad (outlined by yellow dotted lines). Scale bars, 1 mm. (**B**) Sex reversal ratio (percentage of ovaries) of gonads with *Kdm6b*-RNAi#1 and *Kdm6b*-RNAi#2 at 26°C. Gonadal sex was determined by morphological analysis of gonads and the SOX9 stain. GFP, green fluorescent protein. (**C**) Coimmunofluorescence of SOX9 and CTNNB1 (β-catenin) in gonadal sections of 26°C control embryos, three examples of embryos at 26°C after *Kdm6b* knockdown, and 32°C control embryos at stages 21 and 25. (**D**) VASA and CTNNB1 delineate the distribution pattern of germ cells in *Kdm6b*-deficient gonads at stages 21 and 25.

responds rapidly and strongly to *Kdm6b* knockdown at the very beginning of the temperature-sensitive period. Together, these results suggest that *Dmrt1* could be a critical target of KDM6B. As a test of this idea, we knocked down *Kdm6b* and experimentally overexpressed *Dmrt1* in a group of *T. scripta* embryos (fig. S12). Overexpression of *Dmrt1* rescued the male pathway of 16 of 18 (88.9%) *Kdm6b*-deficient 26°C gonads, with both morphology and expression patterns similar to those of control 26°C gonads (Fig. 3C and table S1). SOX9 protein was robustly activated in the primary sex cords of *Kdm6b*-deficient 26°C gonads overexpressing *Dmrt1* (Fig. 3D), and the *Kdm6b* knockdown–induced reduction of *Amh*

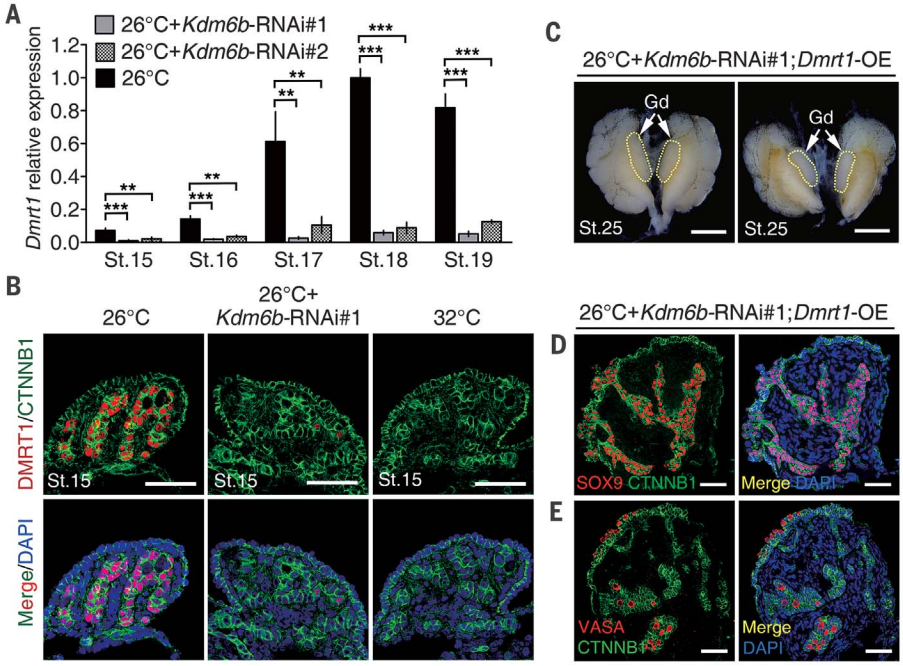
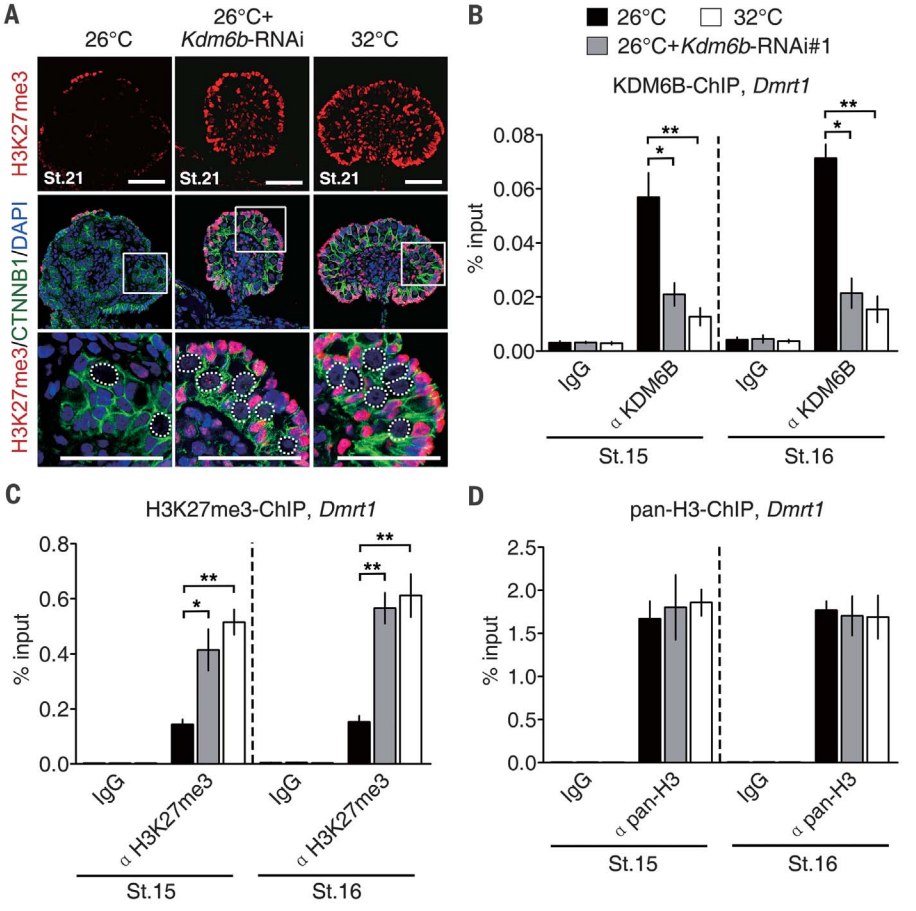


Fig. 3. Knockdown of *Kdm6b* abolishes the expression of *Dmrt1*, but the male pathway can be rescued by overexpression of *Dmrt1*. (**A**) Results of qRT-PCR analysis of *Dmrt1* in gonads from 26°C control embryos, 26°C *Kdm6b*-RNAi#1 embryos, and 26°C *Kdm6b*-RNAi#2 embryos at stages 15 to 19. After normalization to *Gapdh*, the relative expression level in stage 18 gonads at 26°C was defined as 1.0. Data are means ± SD, $n = 3$ biological replicates. $^{**}P < 0.01$; $^{***}P < 0.001$. (**B**) Immunofluorescence of DMRT1 with CTNNB1 in stage 15 gonadal sections from 26°C control, 26°C *Kdm6b*-RNAi#1, and 32°C control embryos. Scale bars, 50 μm. (**C**) Two representative light microscopy images of 26°C *Kdm6b*-RNAi#1 gonads overexpressing *Dmrt1* (*Dmrt1*-OE) at stage 25. Scale bars, 1 mm. (**D** and **E**) Immunofluorescence analysis for SOX9 (D) and VASA (E) with CTNNB1 in stage 25 *Kdm6b*-RNAi#1 gonads at 26°C after forced ectopic expression of *Dmrt1*. Scale bars, 50 μm.

Fig. 4. *Kdm6b* directly regulates H3K27 demethylation at the *Dmrt1* locus. (**A**) Immunofluorescence of H3K27me3 with CTNNB1 in gonadal sections of 26°C control, 26°C *Kdm6b*-RNAi#1, and 32°C control embryos at stage 21. The dotted circles indicate germ cells. Scale bars, 50 μm. (**B**) Quantitative enrichment of KDM6B at the promoter region of *Dmrt1* in 26°C control, 32°C control, and 26°C *Kdm6b*-deficient gonads at stages 15 and 16, as determined by ChIP-qPCR analysis. Signals are shown as a percentage of the input. IgG, immunoglobulin G. (**C** and **D**) Results of ChIP-qPCR assays with antibodies specific for H3K27me3 (C) and pan-H3 (D) at the promoter of *Dmrt1* in 26°C control, 32°C control, and 26°C *Kdm6b*-deficient gonads at stages 15 and 16. Data in (B) to (D) are means ± SD, $n = 3$ biological replicates. $^{*}P < 0.05$; $^{**}P < 0.01$.



and the up-regulation of the female markers *Cyp19a1* and *Foxl2* were all reversed (fig. S13). Male-specific medullar distribution of germ cells was observed in the rescued gonads, although some germ cells remained in the cortex (Fig. 3E). These results indicate that *Dmrt1* functions downstream of *Kdm6b* to initiate the male pathway in *T. scripta*.

We next investigated the molecular mechanism by which *Kdm6b* regulates *Dmrt1* expression. *Kdm6b* was the most highly expressed H3K27 demethylase gene in early male gonads (fig. S14). Immunofluorescence analysis revealed that H3K27me3 was more highly enriched in gonadal cells at 32°C than at 26°C. Knockdown of *Kdm6b* increased the total level of H3K27me3 in gonadal cells at 26°C (Fig. 4A and figs. S15 to S17), consistent with a dominant role for KDM6B in catalyzing demethylation of the repressive mark H3K27me3 and activating target genes.

To investigate a direct link between *Kdm6b* function and *Dmrt1* expression, we next examined KDM6B and H3K27me3 levels at the promoter of *Dmrt1* in *T. scripta* gonads at stages 15 and 16 by performing chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) analyses. Our results show that KDM6B is strongly recruited to the promoter region of *Dmrt1*, with higher enrichment at 26°C than at 32°C (Fig. 4B). Knockdown of *Kdm6b* reduced KDM6B binding to the promoter of *Dmrt1* in gonadal cells (Fig. 4B). Consistently, the enrichment of H3K27me3 in the promoter region of *Dmrt1* was significantly higher at 32°C than at 26°C (Fig. 4C). Knockdown of *Kdm6b* at 26°C led to a significant increase in H3K27me3 levels within the *Dmrt1* locus, without altering histone H3 occupancy (Fig. 4, C and D). In contrast to the ChIP signal at the *Dmrt1* locus, no occupancy of KDM6B or H3K27me3 was found in other early sex-biased genes *Amh*, *Cyp19a1*, *Fdxr*, *Pcsk6*, *Nov*, and *Vwa2* (fig. S18). These results strongly implicate KDM6B as the upstream regulator of the male pathway via catalysis of H3K27 demethylation near the promoter of *Dmrt1*.

This study in *T. scripta* supports a critical role for the chromatin modifier KDM6B in eliminating a repressive mark from *Dmrt1*, a key gene responsible for male sex determination (*18*). An independent RNA sequencing analysis in gonads of the American alligator (*Alligator mississippiensis*) also identified rapid changes in *Kdm6b* expression after shifting eggs from female-producing temperature to male-producing temperature (*19*). Another recent report showed differential intron retention in two members of the jumonji family, *Kdm6b* and *Jarid2*, in adult female dragon lizards that experienced in ovo sex reversal driven by high temperatures (*20*). Sexually dimorphic intron retention of these two genes also was detected in the embryonic transcriptomes of alligators and turtles with temperature-dependent sex determination, but no sex correlation was observed across these species (*20*). Although these findings suggest a reptile-wide role of *Kdm6b* in regulating temperature-dependent sex determination, they also suggest that both evolutionary recruitment to the pathway and the molecular mechanism of action differ across species. Further interspecies comparative experiments considering the broader jumonji family of proteins will be required to unravel this puzzle. Future experiments will also be necessary to determine whether overexpression of *Kdm6b* is sufficient to drive male development at a female-producing temperature.

Another important question is how expression of *Kdm6b* is linked to temperature in *T. scripta*. The gene is not inherently responsive to temperature, as its male-specific expression was initiated at stage 13 in the gonad-mesonephros complexes but not in other embryonic tissues (Fig. 1A). One possibility is an upstream regulator of *Kdm6b* that acts as a gonad-mesonephros complex–specific temperature sensor. This entity could be a gene whose expression is inherently responsive to temperature or a protein whose activity responds to temperature (*17*). Identification of the link between temperature and differential expression of an epigenetic regulator may finally solve the puzzle of how the incubation temperature of the egg can exert its effect on sex determination, a problem that has defied explanation for the 50 years since its initial discovery in reptiles.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/360/6389/645/suppl/DC1
Materials and Methods
Figs. S1 to S18
Tables S1 and S2
References (*21*–*23*)

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Making males and back again

Temperature-dependent sex determination occurs in many reptilian species. An epigenetic mechanism is presumed to be at work, but thus far it has not been identified. Ge *et al.* show that in the red-eared slider turtle, an epigenetic modifier, the histone demethylase KDM6B, binds to the promoter of the dominant male gene to activate male development (see the Perspective by Georges and Holleley). Knock down the expression of KDM6B, and embryos destined to be male turn into females.

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