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REPORT

SEX DETERMINATION

Temperature-dependent sex determination is mediated by pSTAT3 repression of *Kdm6b*

Ceri Weber[1], Yingjie Zhou[2], Jong Gwan Lee[1], Loren L. Looger[3], Guoying Qian[2], Chutian Ge[2]*, Blanche Capel[1]*

In many reptiles, including the red-eared slider turtle *Trachemys scripta elegans* (*T. scripta*), sex is determined by ambient temperature during embryogenesis. We previously showed that the epigenetic regulator *Kdm6b* is elevated at the male-producing temperature and essential to activate the male pathway. In this work, we established a causal link between temperature and transcriptional regulation of *Kdm6b*. We show that signal transducer and activator of transcription 3 (STAT3) is phosphorylated at the warmer, female-producing temperature, binds the *Kdm6b* locus, and represses *Kdm6b* transcription, blocking the male pathway. Influx of Ca^{2+}, a mediator of STAT3 phosphorylation, is elevated at the female temperature and acts as a temperature-sensitive regulator of STAT3 activation.

The sex of many reptiles is determined by the temperature at which the egg develops in the nest. For example, in the red-eared slider turtle, *Trachemys scripta elegans* (*T. scripta*), the embryonic bipotential gonad differentiates into an ovary at 31°C [the female-producing temperature (FPT)] but into a testis at 26°C [the male-producing temperature (MPT)]. However, the molecular mechanisms underlying this temperature-dependent developmental switch have remained elusive (*1*–*4*). A recent advance was made by our discovery that an epigenetic regulator, KDM6B (JMJD3, a histone demethylase), is required for expression of a conserved, male sex–determination gene, *Dmrt1*, at 26°C (*5*, *6*); in the absence of KDM6B, male genes are not activated, and the female pathway is initiated, leading to formation of an ovary (*6*). KDM6B itself is not inherently responsive to temperature, indicating that a thermosensitive regulator upstream must provide the missing link between temperature and sex-specific gene expression (*6*).

One candidate for such a regulator is STAT3 (signal transducer and activator of transcription 3), a transcription factor that controls *Kdm6b* expression (*7*, *8*). Dimerization and translocation of STAT3 to the nucleus are dependent on phosphorylation, which can occur through several signaling pathways in response to environmental or physiological stimuli (*9*–*12*). Our analysis shows that in *T. scripta* embryos, transcript and protein levels of STAT3 are similar at both 26° and 31°C throughout the temperature-sensing window (stages 14 to 20) (Fig. 1A and fig. S1) (*13*–*15*). Immunofluorescence with a phosphospecific antibody showed elevated levels of phosphorylated STAT3 (pSTAT3) at 31°C throughout the embryonic gonad at stages 15 and 17, becoming mostly restricted to the cortex by stage 20 (Fig. 1B). These results were confirmed by means of Western blot analysis of gonad-mesonephric complexes at stages 15 and 17 (Fig. 1C). On the basis of these results, we hypothesized that pSTAT3 binds the *Kdm6b* locus at 31°C and acts as an inhibitor of *Kdm6b* transcription.

To test whether pSTAT3 binds the *Kdm6b* locus, we collected gonads at stage 16 and performed chromatin immunoprecipitation (ChIP) for pSTAT3 followed by quantitative polymerase chain reaction (qPCR) using primers for two conserved pSTAT3 binding sites in the 5′ end of the *Kdm6b* locus (fig. S2). pSTAT3 is enriched at the *Kdm6b* locus at 31°C, and its binding is blocked by HO-3867, an inhibitor of STAT3 phosphorylation and DNA binding (Fig. 2A).

To determine whether pSTAT3 functions as a repressor of *Kdm6b*, we explanted stage-14 whole gonad-mesonephros complexes (GMCs) to organ culture at 31° or 26°C. GMCs were exposed to one of two pSTAT3 inhibitors with different mechanisms of action, HO-3867 or NSC 74859 (S31-201, inhibitor of STAT3 phosphorylation and dimerization), and collected after 24 hours for analysis (*16*, *17*). Levels of pSTAT3 declined in response to both inhibitor treatments, according to immunofluorescence and Western blot analysis (Fig. 2, B and C, and fig. S3A). Reverse transcription followed by qPCR (RT-qPCR) showed that expression of both *Kdm6b* and its downstream target *Dmrt1* was up-regulated at 31°C after exposure to either inhibitor of STAT3 phosphorylation (Fig. 2D). *Kdm6b* expression was not significantly affected by either pSTAT3 inhibitor at 26°C (fig. S3B). This supports a model in which pSTAT3 represses *Kdm6b* transcription at the FPT.

To determine whether pSTAT3 inhibition had a similar effect in vivo, we injected increasing doses of HO-3867 (25, 50, and 100 μM) into eggs at stage 14 and collected gonads at stages 16 and 21 (Fig. 3A). Consistent with organ culture results, pSTAT3 inhibition led to up-regulation of *Kdm6b* and *Dmrt1* in eggs incubated at 31°C (Fig. 3B). Consequently, 16 of 23 (69.6%) HO-3867–treated eggs exhibited

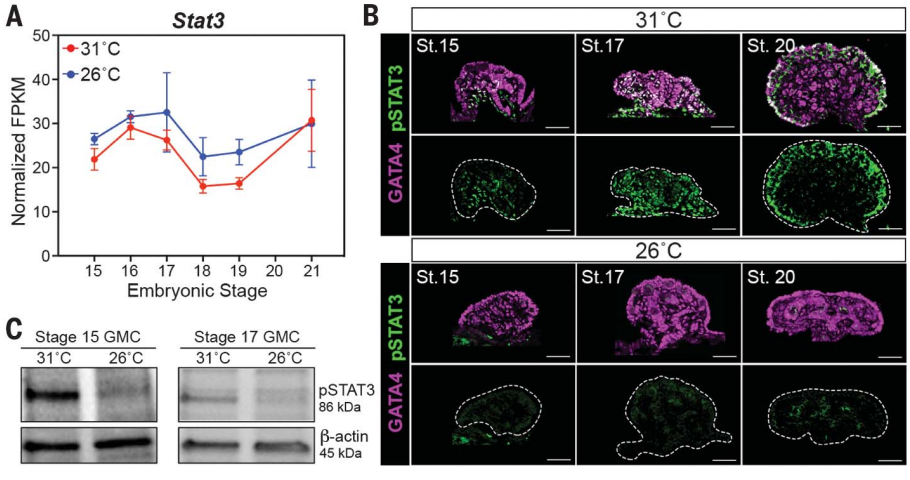

Fig. 1. STAT3 phosphorylation is temperature-dependent and sexually dimorphic. (**A**) RNA-sequencing measurements of STAT3 expression over the temperature-sensing period at both 26°C (blue) and 31°C (red) (*15*). FPKM, fragments per kilobase of exon per million fragments mapped. (**B**) Immunofluorescent images of pSTAT3 (green) and GATA4 (magenta; somatic gonad marker) in gonadal cross sections from embryos at stages 15, 17, and 20. pSTAT3 expression is nuclear and restricted to the sex cords and cortical domain. Scale bars, 50 μm; $n > 3$ biological replicates. (**C**) Western blot analysis of pSTAT3 levels at stage 15 and stage 17 in GMCs, with β-actin control; $n > 3$ biological replicates.

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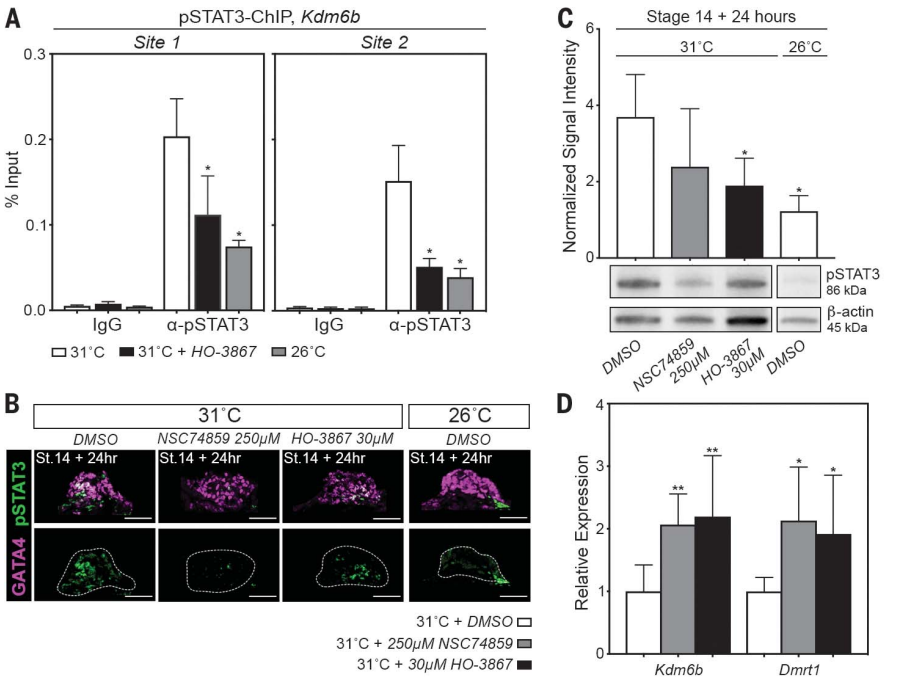

Fig. 2. pSTAT3 binds Kdm6b at 31°C and represses transcription. (**A**) ChIP-qPCR analysis shows enrichment of bound pSTAT3 at two sites in the *Kdm6b* locus in gonads at 31°, 26°, and 31°C with HO-3867 treatment. Values are shown as a percentage of the input. IgG, immunoglobulin G control; $n = 3$ pooled biological replicates; data are means ± SD. (**B**) Immunofluorescent images of pSTAT3 (green) and GATA4 (magenta) in gonad sections from cultured GMCs treated with dimethyl sulfoxide (DMSO), NSC 74859, or HO-3867 at 31° or 26°C. Scale bars, 50 μm. (**C**) Western blot analysis of pSTAT3 levels in GMCs treated with DMSO, NSC 74859, or HO-3867 at 31° or 26°C. Western blot band intensity was quantified after normalization to β-actin. (**D**) mRNA levels of *Kdm6b* and *Dmrt1* in GMCs cultured at 31°C with DMSO, NSC 74859, or HO-3867. Expression levels of DMSO-treated GMCs were set as 1 after normalization to *Gapdh*. DMSO, $n = 9$ biological replicates; HO-3867, $n = 5$ biological replicates; NSC 74859, $n = 6$ biological replicates. Data are means ± SD; $^*P < 0.05$, $^{**}P < 0.005$, Mann-Whitney nonparametric *t* test.

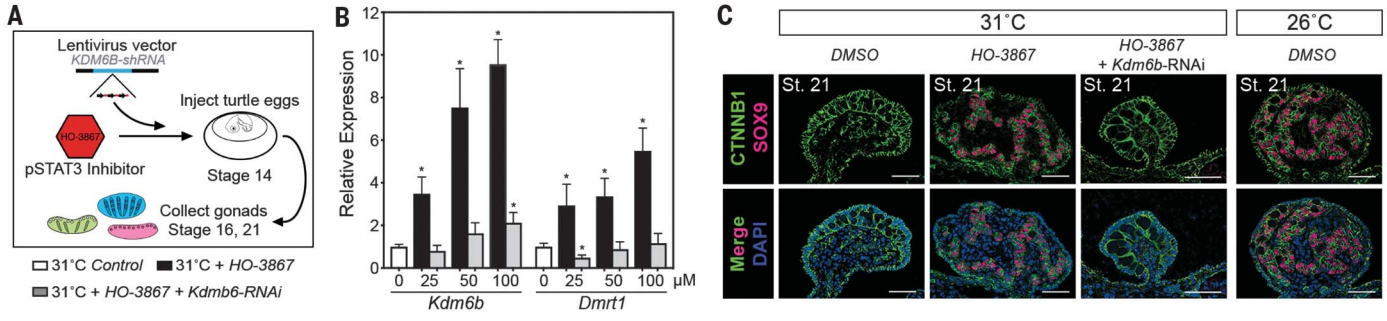

Fig. 3. The switch from the ovary to the testis pathway induced by pSTAT3 inhibition can be reversed by knockdown of *Kdmb6*. (**A**) Experimental overview. Eggs raised at 31°C were injected at stage 14 with pSTAT3 inhibitor HO-3867 or co-injected with HO-3867 and *Kdm6b*-RNAi and collected at stage 16 and stage 21 to capture the effect of pSTAT3 inhibition early and late in the sex-determination window. (**B**) mRNA levels of *Kdm6b* and *Dmrt1* in stage-16 gonads from 31°C eggs injected with increasing concentrations of HO-3867, or HO-3867 and *Kdm6b*-RNAi. Control 31°C gonads were set as 1 after normalization to *Gapdh*. Data are means ± SD; $n = 3$ pooled biological replicates. $^*P < 0.05$, Mann-Whitney nonparametric *t* test. (**C**) Immunofluorescence of SOX9 (pink) and CTNNB1 (β-catenin, green) in stage-21 gonads from 31°C eggs injected with 100 μM HO-3867 alone or in combination with *Kdm6b*-RNAi compared with gonads from 26°C eggs. Scale bars, 50 μm. DAPI, 4′,6-diamidino-2-phenylindole.

an ovary-to-testis shift in sexual trajectory (table S1), as determined by the ectopic expression of SOX9 protein in gonads at stage 21 (Fig. 3C). However, the up-regulation of *Dmrt1* after inhibition of pSTAT3 at 31°C could be prevented by knocking down *Kdm6b* expression with *Kdm6b*–RNAi (RNA interference) (Fig. 3B), which restored ovarian development in 92.9% (13 of 14) of HO-3867–treated gonads at 31°C (Fig. 3C and table S1). These data further support the model that pSTAT3 binds the *Kdm6b* locus to repress activation of the male pathway at 31°C.

Phosphorylation of STAT3 can be regulated by calcium signaling pathways that respond to environmental signals, including temperature (*18*). To investigate a possible relationship between temperature-driven calcium signaling and sex determination, we derived primary cells from stage 15 *T. scripta* gonads. A single-wavelength calcium indicator dye, Cal-520 acetoxymethyl ester (Cal-520 AM) (AAT Bioquest) (*19*, *20*), was introduced into these cells in culture to measure the relative calcium levels at either 26° or 31°C (diagrammed in fig. S4). Cells grown at 31°C showed more fluorescent activity than cells at 26°C, suggesting that Ca^{2+} influx is elevated at FPT (Fig. 4A). Cells were imaged live as the temperature was quickly shifted from 26° to 31°C. Significantly higher fluorescence was recorded almost immediately after the temperature was increased (Fig. 4B, figs. S5A and S6, and movie S1). This effect could be mimicked by exposing cells at 26°C to the calcium ionophore A23187 (Fig. 4C and fig. S5B). Conversely, cells treated with the calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)–AM reported diminished levels of fluorescence over time, despite a shift to 31°C (Fig. 4C and fig. S5B). Western blot analysis of A23187- or BAPTA-AM–treated cells showed that exposure to the ionophore A23187 drives phosphorylation of STAT3 at 26°C, whereas chelation of calcium with BAPTA-AM during the shift from 26° to

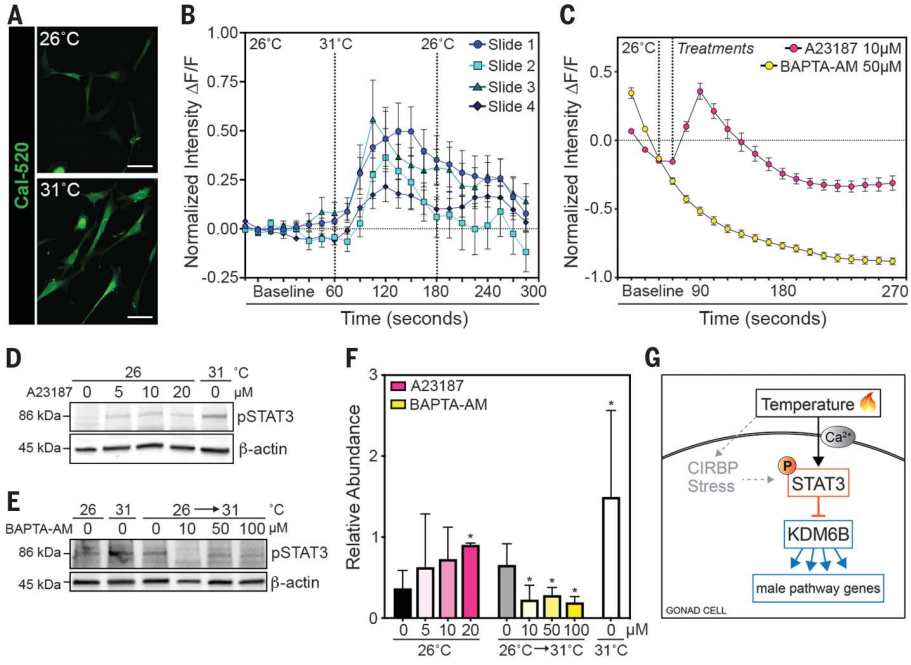

Fig. 4. Intracellular [Ca^{2+}] increases at 31°C are associated with temperature-dependent phosphorylation of STAT3. (**A**) Gonad-derived primary cells display higher levels of fluorescence at 31°C. (**B**) Fluorescence (*F*) was recorded in cells exposed to a temperature shift from 26° to 31°C. Data are means ± SE; *n* = 4 slides. (**C**) Cells were stimulated with ionophore A23187 and maintained at 26°C or treated with chelator BAPTA-AM and then shifted to 31°C. (**D**) pSTAT3 was present in primary cells at 26°C after treatment with increasing concentrations of A23187 for 5 min. (**E**) STAT3 phosphorylation was diminished when cells at 26°C were treated with increasing concentrations of BAPTA-AM and then switched to 31°C for 5 min. (**F**) pSTAT3 levels in treated cells were compared with control cells grown at 26° or 31°C. Western blot band intensity was quantified after normalization to β-actin. Data are means ± SD; *n* = 3 biological replicates. (**G**) A molecular model for temperature-dependent sex determination in *T. scripta*. Warm temperatures initiate a rise in intracellular calcium that promotes STAT3 phosphorylation. pSTAT3 binds *Kdm6b* to repress activation of *Dmrt1* and the male pathway. Temperature may also activate CIRBP and/or stress response pathways, which can also activate STAT3.

31°C leads to diminished activation of STAT3 (Fig. 4, D to F). Collectively, these results show that higher calcium levels at 31°C promote STAT3 phosphorylation.

Taken together, our findings support a new model for sex determination in *T. scripta* (Fig. 4G). According to this model, at warmer temperatures (31°C), ovary development is initiated by a robust influx of calcium into gonadal cells. This promotes phosphorylation of STAT3 and repression of *Kdm6b*, a required activator of the testis-determining pathway. A critical question is therefore how calcium flux is regulated by temperature. Potential effectors for this step are transient receptor potential (TRP) cation channels, well-known environmental sensors that can initiate calcium signaling in response to temperature stimuli (*21*). TRP channel activity is reported to activate STAT3 in mammalian cell systems and may mediate thermoregulatory responses during inflammation (*18*). In another reptile with temperature-dependent sex determination, the American alligator, pharmacological manipulation of TRPV4 led to changes in expression of male pathway genes *Sox9* and *Amh* (*22*). In *T. scripta* gonads, several TRP channels were up-regulated early in the temperature-sensitive period at 31°C (*15*), including TRPV4, and this may contribute to additional biases in Ca^{2+} flux. When conditional approaches become feasible in *T. scripta*, we anticipate that a targeted knockdown of candidate cation channels individually and in combination will be informative. Although TRP channel activity and intracellular calcium are indicated in STAT3 activation, it is unclear whether STAT3 is phosphorylated through canonical signaling (Janus kinase/STAT), other kinases such as calcium/calmodulin-dependent protein kinase II (CaMKII) or Src, or even TRP channels directly (*18*, *23*, *24*). Although we propose a calcium-mediated mechanism, it is possible that other well-characterized thermoregulatory responses such as heat shock protein activity also contribute to STAT3 phosphorylation and sex determination in *T. scripta* (*25*). Sex determination is likely a cumulative process, and other conserved factors—such as anti-Müllerian hormone (AMH), doublesex and mab-3 related transcription factor 1 (DMRT1), (sex determining region Y)–box 9 (SOX9), or aromatase—likely feed into the Ca^{2+}-pSTAT3-*Kdm6b* regulatory loop to stabilize ovary or testis development.

Any model of temperature-dependent sex determination must account for natural nests experiencing oscillating temperatures, unlike the constant temperature experienced by eggs in the laboratory. Broad temperature fluctuations are sufficient to feminize developing turtle embryos, indicating that regular exposures to warmer temperatures are sufficient to initiate and maintain female development (*26*). Through temperature-shifting experiments, Wibbels *et al.* hypothesized that the magnitude and duration of temperature exposure exerts an effect on sex determination by driving the accumulation of a sex factor in sufficient quantities; a pulse of 26°C MPT lasting at least three embryonic stages or a pulse of 31°C FPT lasting 1.5 embryonic stages was sufficient to affect sex determination (*27*). Our data show that a rapid, calcium-mediated response to 31°C leads to phosphorylation of STAT3. Because STAT3 is reported to be stable for at least 8 hours (*28*), intermittent exposure to high temperature may be sufficient to repress the male pathway in the face of fluctuating temperatures.

In other organisms, a variety of physiological changes other than temperature can regulate the sex-determining pathway, but we argue that these may nevertheless converge on the differential activation of STAT3 and its downstream target, *Kdm6b*. For example, in sequentially hermaphroditic fish, cortisol and the hypothalamus-pituitary-adrenal axis are thought to mediate the social and hormonal changes that transform the identity of the gonad (*29*). STAT3 can be activated by cortisol through the adenosine 3′,5′-monophosphate (cAMP)–cAMP-dependent protein kinase (PKA)–Src pathway (*11*, *24*). Proopiomelanocortin (POMC)—the precursor to adrenocorticotropic hormone (ACTH), which stimulates the release of cortisol—is up-regulated in temperature-driven sex-reversed female Australian bearded dragons, suggesting that POMC-mediated stress modulates gonadal fate in some reptiles (*30*). Other pathways involved in homeostasis have also been implicated in STAT3 activation. Cold-inducible RNA-binding protein (CIRBP) is activated in response to a number of environmental stimuli, including hypothermia (<32°C), and has been associated with female specification in another turtle species with temperature-dependent sex determination (*31*). CIRBP is a reported activator of STAT3 by way of a number of signaling pathways (*12*), including calcium and TRPV4 signaling (*32*). Taken together, these observations support a unifying concept in which pSTAT3 repression of the epigenetic

regulator *Kdm6b* is a conserved cassette in multiple environmental sex-determination networks.

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SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/368/6488/303/suppl/DC1
Materials and Methods
Figs. S1 to S6
Tables S1 to S3
References (*33*, *34*)
Movie S1

View/request a protocol for this paper from *Bio-protocol*.

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Science

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How egg temperature sets sex

In many reptiles, sex is determined by nest temperature during egg incubation. Temperature regulates the expression of an epigenetic modifier gene called *Kdm6b*, which is responsible for testis development. However, the molecular connection between temperature and sex-specific expression of this factor was previously unknown. Weber *et al.* have identified a link between temperature and the activation of a key regulator of *Kdm6b* called signal transducer and activator of transcription 3 (STAT3). After an influx of $Ca2+$ at the warmer, female-producing temperature, STAT3 is phosphorylated and silences *Kdm6b* transcription to repress testis development.

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