Trpv4 mediates temperature induced sex change in ricefield eel

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Abstract

The ricefield eel (*Monopterus albus*), an economically important aquaculture species in China, is the only freshwater teleost fish that exhibits protogynous hermaphroditism. While progress has been made toward understanding the sex determination and differentiation of this species, the underlying mechanisms remain elusive. Here we show that warm temperature promotes gonadal transformation by up-regulating testicular differentiation genes such as *dmrt1/sox9a* in ovaries. Trpv4, a Ca^{2+}-permeable cation channel expressed in gonads, is highly sensitive to ambient temperature and mediates warm temperature-driven sex change of ricefield eel. In female fish reared at cool temperature, injection of Trpv4 agonist into the ovaries leads to significant up-regulation of testicular differentiation genes, and in female fish exposed to warm temperature, Trpv4 antagonist administration or *trpv4* siRNA knockdown inhibits temperature-induced male gene expression. pStat3 signaling is downstream of Trpv4 and transduces Trpv4-controlled calcium signaling into the sex determination cascades. Inhibition of pStat3 activity prevents the up-regulation of male genes by warm temperature treatment and ovarian injection of Trpv4 agonist, whereas activation of pStat3 is sufficient to induce the expression of male genes, even in the presence of Trpv4 antagonist. pStat3 binds and activate *jmjd3*/*kdm6b*, an activator of the male gene *dmrt1*. Consistently, ovarian injection of Kdm6b inhibitor blocks the up-regulation of male genes by warm temperature treatment. We propose that environmental factors such as warm temperature promote gonadal transformation of ricefield eel via the Trpv4-pStat3-Kdm6b axis. Our results provide new insights into the molecular mechanism underlying natural sex change of ricefield eel, which will be useful for sex control in aquaculture.

Key words: ricefield eel, *trpv4*, pStat3, *jmjd3*/*kdm6b*, sex change

Highlights

- Warm temperature promotes gonadal transformation of ricefield eel
- Trpv4 links environmental temperature and the sex determination pathway
- pStat3 is downstream of Trpv4-controlled calcium signaling
- pStat3 binds and activates *kdm6b/jmjd3*

Introduction

Sex determination in animals is intriguing and fascinating. In mammals, sex is determined genetically (genotypic sex determination, GSD). In fish and reptiles, however, sex regulators are diverse. Their sex can be influenced by various environmental factors, including temperature, pH, the breeding density and social status (Honeycutt et al. 2019; Mei and Gui, 2015; Todd et al., 2019), the so-called environmental sex determination (ESD). The temperature-dependent sex determination (TSD) is one of the best studied forms of ESD. In red-eared slider turtle (*Trachemys scripta*), American alligator (*Alligator mississippiensis*) and Atlantic silverside (*Menidia menidia*), sex is determined solely by the temperature during the thermosensitive period of embryogenesis. In Australian central bearded dragon (*Pogona Vitticeps*) and Nile tilapia (*Oreochromis niloticus*), which display GSD, temperature can override the genetic materials to control the gonadal sex differentiation (Deveson et al., 2017; Holleley et al., 2015). Analysis of expression data during embryogenesis of normal ZWf females and temperature sex reversed ZZf females has provided important insights into gene-driven and temperature-driven female development in the bearded dragon (Whiteley et al., 2021). Irrespective of TSD or GSD+TE (temperature effects), the downstream components are fairly conserved, including the epigenetic factors such as *jmjd3*/*kdm6b* and the JAK/STAT signaling pathway (Lu et al., 2025; Martinez-Pacheco et al., 2024; Weber et al., 2020; Whiteley et al., 2020; Wu et al., 2024).

Most vertebrates, including the TSD reptiles, exhibit gonochorism. However, approximately 6% of fish species exhibit hermaphroditism, including protandrous, protogynous, and bidirectional hermaphroditism (Todd et al., 2016). The majority of them are marine fish, appearing in 27 families (Muncaster et al., 2013; Peng et al., 2020; Shao et al., 2014; Todd et al., 2019). Compared to marine fish, sex change in freshwater fish is very rare. The ricefield eel, also called Asian swamp eel, which belongs to the family Synbranchidae in the Synbranchiformes, is by far the only freshwater fish that exhibits natural sex change in China. The fish begins life as a female and then develops into a male through an intersex stage. This teleost fish is emerging as an important model animal for studying sex determination and differentiation as well as adaptive evolution (Ji et al., 2001). As early as the Ming Dynasty in ancient China in 1578, pharmacist Shi-Zhen Li has described the medicinal value of ricefield eel in treatment of human diseases in his famous

pharmacy monograph, the Bencao Gangmu, also called "Compendium of Materia Medica" (Cheng and Zhou, 2022). Nowadays, the ricefield eel has been developed as one of the most important economical fish in freshwater aquaculture in China, with annual production exceeding 300,000 tons (Zhou and Gui, 2016). Unfortunately, the population has declined rapidly in the wild due to overfishing. Although great progress has been made in artificial breeding, the reproductive mode of ricefield eel severely affects the sex ratio, and decreases the productivity of broodstock. To date, quality fries for aquaculture industry are still obtained from the wild fishing. Thus, the elucidation of the mechanisms underlying the sex change is urgent, which will aid in developing techniques for sex control that would break the bottleneck in aquaculture industry (Wu et al., 2019; Chen and Zhou, 2021).

The life history of ricefield eel implies that environmental factors initiate and promote the gonadal transformation via epigenetic mechanisms. Consistently, histone demethyltransfearse/methyltransfearse genes such as *kdm6b/kmt2* and DNA methylation enzyme genes such as *dnmt1/2* were dynamically expressed throughout the sex change process, and the expression levels of the master sex determination/differentiation genes are closely correlated to the levels of DNA and histone methylation, which can be impacted by environmental exposure (Fan et al., 2021, 2022; Jiang et al., 2021; 2022; Hu et al., 2022; Wang et al., 2020). However, these epigenetic regulators are not inherently responsive to the environmental cues, implying that certain molecular sensors exist and serve as the link between environmental stimuli and the sex determination pathway. We have recently proposed that there is a temperature induced sex reversal (TISR) mechanism in ricefield eel, similar to that of embryonic bearded dragon (*P. Vitticeps*) (Zhang et al., 2025). Isolated ovarian tissues are responsive to temperature stimuli, suggesting that the initial reception of temperature is triggered by endogenous sensors such as Transient Receptor Potential (Trp) channels. While the Ca^{2+}-permeable, non-selective cation channel Trpv4 has been identified as a potential thermosensor, how Trpv4-mediated Ca^{2+} influx are transduced into the sex determination cascades remains to be determined.

Recent studies have shown that signal transducer and activator of transcription 3 (STAT3) is phosphorylated by warm temperature-evoked Ca^{2+} influx, and then binds and transcriptionally regulates *kdm6b*, thereby initiating the female pathway by repressing the male sex determination gene *dmrt1* (Weber et al., 2020). Based on the

observation from our RNA-seq (RNA-sequencing) data that *trpv4*, calcium handling genes and *kdm6b* were higher expressed in ovotestes than in ovaries, we hypothesized that Trpv4 may bridge environmental temperature and the sex differentiation pathway via a mechanism similar to the TSD turtle. By using small molecule agonist and antagonist of Trpv4 as well as siRNA-mediated knockdown of *trpv4*, we provided solid evidences that temperature-evoked Trpv4 activity promotes the expression of testicular differentiation genes in ovaries via the downstream Ca^{2+}-pStat3-Kdm6b axis.

Results

Warm temperature promotes gonadal transformation

Natural populations of ricefield eel are widely distributed across East Asia and Southeast Asia. Previous work has shown that at the onset of sex change, females from different geographic populations vary in age, body weight and length. It is around 16 cm long (1.5-year-old) in Indonesia (Liem, 1963), and 20 cm in Hainan and Guangzhou areas of south China (Chan and Philips, 1967; Wang and Zeng, 2006；Wang et al., 2008), 30 cm (2-year-old) in Wuhan area of central China, and 35-40 cm (3-year-old) in Tianjin area of north China (Fan et al., 2017; Liu and Wang, 1987). The average annual temperature in Hainan, Guangzhou, Wuhan and Tianjin areas is approximately 25, 22, 17, and 13 ℃, respectively (Figure 1A). Specifically, in the Wuhan area (Hubei province, China), the reproductive season of ricefield eels usually runs from May to August which are the warmest months, when the average monthly temperature is 25-35 ℃ (Figure S1A). Immediately after spawning in the reproductive season, the females (2-year-old) may undergo extensive ovarian tissue degeneration and physiological change, which leads to an irreversible commitment to becoming male via an intersex stage (Figure S1B). Thus, the onset of sex change of ricefield eels is closely related to the external environment, in particular the warm temperature.

The observations prompted us to hypothesize that warm temperature promotes the gonadal transformation of ricefield eel. To directly test this, long term temperature experiments were performed using female ricefield eels collected from Wuhan Area, Hubei province (Figure 1B). 2-year old females (length = 25-35 cm) were randomly divided into two groups, and reared at 25 ℃ (cool temperature, CT) and 33 ℃ (warm temperature, WT), for a period of 6-8 months. The gonadal sex of each fish was determined by H&E staining and expression analysis of sex-biased genes (Figure 1C).

The average body length and weight of ricefield eels was comparable between the WT group and the CT group (data not shown). In CT group at day 180, 80% gonads were ovaries, 20% were ovotestes. In WT group (33 ℃), however, 35% gonads were ovaries, 65% were ovotestes (Figure 1D). The percentage of sex reversing animals was significantly higher at warm temperature than at cool temperature. The results indicated that warm temperature promotes gonadal transformation of ricefield eel.

***trpv4* is highly responding to environmental temperatures**

It has been shown that *trpv4* mRNA levels in gonad are elevated by warm temperature treatment, and that Trpv4 cation channel activity promotes male gene expression in TSD alligator and ricefield eel (Huang et al., 2024; Yatsu et al., 2015; Zhang et al., 2025). We hypothesized that gonadal-expressed Trpv4 functions as a thermosensor that perceives/detects the environmental temperature cues. The results of qPCR experiments showed that *trpv4* was higher expressed in gonadal tissues than in non-gonadal tissues, with highest expression levels in testis (Figure 2A). RNA in situ hybridization (ISH) experiments confirmed that *trpv4* levels increased from ovaries to testes (Figures 2B), implying that it was functionally associated with testicular development.

In cultured primary ovarian explants, *trpv4* was one of the most up-regulated *trp* genes by warm temperature treatment (Figure 2C). Temperature-shifting experiments showed that *trpv4* was highly sensitive to temperature change (Figure 2D), displaying a pattern similar to the testicular differentiation genes such as *dmrt1/sox9a*, but inverse to that of the ovarian differentiation genes such as *cyp19a1a/foxl2* (Figure S2A). *trpv4* was elevated as early as 4 hours post warm temperature treatment (Figure 2E), accompanied by increased calcium signals (data not shown). The results suggested that exposure to warm temperature causes a rapid increase in cytosolic Ca^{2+} concentration through Trpv4, similar to the embryonic dragon (Whiteley et al., 2021). This was also supported by our RNA-seq data that *trpv4* and many genes involved in Ca^{2+} transport and sequestration were up-regulated in ovotestes compared to ovaries (Zhang et al., 2025) .

While *trpv4* is highly responding to temperature changes in cultured ovarian cells, it is not known whether this was the case in vivo. To explore this, female fish were transiently reared at cool temperature (25 ℃) for 1 day, and then shifted to warm temperature (34 ℃) for 10 days (Figure 2F). *trpv4* mRNA in ovaries was already

elevated on day 1 after shifting to 34 ℃ (Figure S2B), and its levels progressively increased over time, exhibiting a pattern similar to that of testicular differentiation genes. To understand in more detail of the role of *trpv4*, we studied its expression in ovaries by performing ISH experiments (Figure 2G). At 26 ℃, *trpv4* signals were detected in ovarian somatic cells around the immature previtellogenic oocytes and mature oocytes, as well the interstitial cells (Figure S2C). After exposure to 34 ℃, *trpv4* signals remained in those cells, but became stronger over time (Grier et al., 2016; Nostro et al., 2003; Yamamoto et al., 2024). The observation suggested that *trpv4* expressing somatic cells may play an important role in gonadal transformation in response to warm temperature.

Temperature-induced male gene expression depends on Trpv4

The expression patterns of *trpv4* and male biased genes prompted us to ask whether Trpv4 in ovaries functions as a thermosensor and transduces the temperature cues into the sex determination cascades. To investigate this, animal experiments were performed by injecting into ovaries with small molecules RN1734 and GSK1016790A, a Trpv4 specific antagonist and agonist, respectively (Liu et al., 2021). The experimental females were reared and divided into 4 groups based on the combination of temperatures and the injected small molecules: 25 ℃+ DMSO; 25 ℃+ GSK1016790A; 34 ℃+ DMSO; 34 ℃+ RN1734 (Figure 3A). 3-10 days post injection, the gonads were isolated for the subsequent analyses.

The qPCR results showed that compared to cool temperature, warm temperature exposure increased the expression of testicular differentiation genes, accompanied by moderately decreased expression of ovarian differentiation genes (Figure 3B). Injection of 10 μM RN1734 blocked the up-regulation of male/testicular differentiation genes by warm temperature treatment, whereas injection of 0.1 μM GSK1016790A at 25 ℃ was sufficient to induce the expression of male/testicular differentiation genes to a extent similar to warm temperature treatment, at the expense of female genes. The effects small molecules on gene expression were dose dependent (Figure S3A). Similar results were observed at the protein levels, as revealed by Western blot (WB) and Immunofluorescence (IF) experiments (Figures 3C-E). We also repeated the experiments using cultured ovarian explants and/or cells, which produced similar results (Figure S3B-D). The observations indicated that the ion

channel Trpv4 is sufficient and necessary to induce the expression of male sex genes in ovaries.

To further demonstrate this, *trpv4* siRNA was injected into the ovaries. *trpv4* expression was markedly down-regulated by 0.1 μM siRNA injection (Figure 4A). Importantly, siRNA injection led to decreased expression of testicular differentiation genes that were up-regulated by warm temperature exposure, and increased expression of ovarian differentiation genes that were repressed by warm temperature treatment. Similar results were observed at the protein levels as revealed by WB experiments (Figures 4B).

Taken together, we concluded that warm temperature-induced male gene expression is dependent on the ion channel activity of Trpv4.

pStat3 signaling is downstream of Trpv4

We went on to ask how Trpv4-controlled Ca^{2+} flux was interpreted and transduced into the sex determination cascades. In TSD turtles and the central bearded dragon, Stat3/4 are activated through phosphorylation by temperature-evoked calcium signaling (Weber et al., 2020; Whiteley et al., 2021). Based on our RNA-seq data, s*tat3*, and the Jak/Stat3 pathway target genes such as *socs3*, and *egr1/2*, were significantly up-regulated in early ovotestes compared to ovaries (Figure 5A). We therefore hypothesized that pStat3 signaling is downstream of Trpv4-contolled calcium influx to promote the expression of male pathway genes in ovaries during sex change of adult ricefield eel. In fact, several lines of evidences were in favor of this hypothesis. First, activation of Trpv4 ion channel by GSK1016790A at cool temperature led to elevated pStat3 levels and calcium signals in ovarian explants, similar to that by higher temperature treatment, and inhibition of Trpv4 ion channel by RN1734 at warm temperature decreased pStat3 levels and calcium signals (Figure 5B-C). Second, the levels of pStat3 were gradually increased from ovaries to testes in wild-caught ricefield eels, along with the male factor Amh (Figures 5D; Figures S5A-B). Importantly, pStat3 signals were detected in the gonadal somatic cells around the oocytes and interstitial cells, analogous to that of *trpv4* (Figure 2G; Figure S2C). These observations suggested that endogenous Trpv4 cell autonomously controls phosphorylation of Stat3 via calcium signaling in gonadal somatic cells.

To explore the function of the pStat3 signaling, animal experiments were performed by injecting into ovaries with small molecules HO-3867 or Colivelin. HO-3867, a curcumin analogue, is a selective pStat3 inhibitor, which blocks pStat3

activity by directly binding to Stat3 DNA binding domain, and Colivelin is a potent synthetic peptide activator of pStat3, which increases pStat3 levels by acting through the GP130/IL6ST complex (Wu et al., 2024). The injected females were divided into 4 groups based on the temperatures and the small molecules injected: 26 ℃+ DMSO; 26 ℃+ Colivelin; 34 ℃+ DMSO; 34 ℃+ HO-3867 (Figure 6A). 3-10 days post injection (dpj), the ovaries were isolated and subjected to the subsequent analyses.

Injection of 30 μM HO-3867 blocked the up-regulation of testicular differentiation genes by warm temperature exposure, whereas injection of 50 μM Colivelin at 26 ℃ was sufficient to induce the expression of these genes to a extent similar to warm temperature treatment, at the expense of ovarian differentiation genes (Figures 6B). Similar results were also observed at the protein levels as revealed by IF experiments (Figures 6C-D). We also repeated the experiments using ovarian explants and cells, which produced similar results (Figure S6A-B). We concluded that warm temperature-induced male gene expression is dependent on pStat3 activity.

To functionally demonstrate that pStat3 signaling is downstream of Trpv4, rescue experiments were performed by injecting into ovaries with individual and combined small molecules. The female animals were divided into 6 groups based on the temperatures and the small molecules injected: 25 ℃+ DMSO; 25 ℃+ GSK1016790A; 25 ℃+ GSK1016790A+HO-3867; 34 ℃+ DMSO; 34 ℃+ RN1734; 34 ℃+ RN1734+ Colivelin. 3-5 days post injection, the ovaries were isolated and subjected to the qPCR analysis. The results showed that up-regulation of testicular differentiation gene expression by the administration of Trpv4 agonist at 25 ℃ was abolished by the treatment with pStat3 inhibitor, and that expression of male genes inhibited by Trpv4 antagonist treatment at 34 ℃can be partially restored by the injection with pStat3 agonist (Figure 6E). Similar results were obtained using in vitro cultured ovarian explants (Figure S6C). Based on the results, we concluded that Trpv4 promotes male sex gene expression in ovaries via activation of the pStat3 signaling.

pStat3 binds and activates *kdm6b*

Kdm6b has been shown to a male pathway promoting factor in TSD/TISR reptiles and Nile tilapia, which can bind and activate *dmrt1* via removing the repressive histone mark H3K27me3 (Chen et al., 2024; Ge et al, 2018; Lu et al., 2025; Yao et al;, 2024). And pStat3 transcriptionally regulates the expression of *kdm6b* by directly binding to its upstream DNA motifs (Wu et al., 2024; Weber et al., 2020). We

reasoned that pStat3 activates *kdm6b* in ovaries of ricefield eel. When analyzing -5 kb promoter sequences upstream the TSS of the *kdm6b* gene, we found that there were three conserved pStat3 binding sites (TTCnnnGAA) (Figure 7A). Chromatin immunoprecipitation (ChIP) experiments using pStat3 antibodies were performed, and the results showed that pStat3 levels were significantly higher at *kdm6b* promotor in ovaries of females reared at warm temperature than at cool temperature. Importantly, the binding was blocked in the presence of pStat3 inhibitor HO-3867 (Figure 7B).

kdm6b mRNA was expressed in immature oocytes and mature oocytes in ovaries (Figure 7C), and was induced by warm temperature exposure (Figure 3B), analogous to that of *trpv4*. The expression of *kdm6b* was elevated by GSK1016790A or Colivelin injection at 25 ℃, and was down-regulated by RN1734 or HO-3867 injection at 34 ℃(Figures 3B; 6B; S3B; S6A). Thus, the *kdm6b* gene displayed a similar expression pattern to that of the testicular differentiation genes (Figure 6B), which strongly supported that *kdm6b* is a male pathway gene downstream of Trpv4-pStat3.

If *kdm6b* is a downstream of Trpv4-pStat3 to control the expression of *dmrt1*, inhibition Kdm6b demethyltransferase activity should prevent up-regulation of testicular differentiation genes by warm temperature treatment, similar to HO-3867 and RN1734 treatment. 0.5 μM GSK-J4, an Kdm6b specific inhibitor, was injected into the ovaries of ricefield eels, and the expression of sex biased factors were examined by qPCR and IF analyses. The results showed that GSK-J4 injection significantly down-regulated the expression of testicular differentiation factors at the expense of ovarian differentiation factors (Figures 6C-D). The observations indicated the presence of the Trpv4-pStat3-Kdm6b axis that links the environmental temperature and the sex determination cascades (Figure 7D).

Discussion

Sex control breeding in ricefield eel is still challenging, mostly due to the incomplete understanding of the biology underlying the sex determination and differentiation in this species. In this work, we provided solid evidences that warm temperature promotes the sex change of adult ricefield eel, and that the thermosensor Trpv4 is the molecular link between environmental temperature and the sex determination

pathway. Our results support a model that temperature-driven sex change is via the Trpv4-pStat3-Kdm6b axis. In this model, warm temperature exposure leads to increased calcium influx via Trpv4, which promotes phosphorylation of pStat3 and activation of *kdm6b/dmrt1* in the ovaries, eventually resulting in transformation of ovary to testis. Our work, for the first time, provides the mechanistic explanation of how natural sex change occurs in ricefield eel, and expands temperature-induced sex reversal from reptiles to the teleost. This work provides a new paradigm to study natural sex change of ricefield eel, which should be also useful for other teleost with TISR, including the marine fish.

The transient receptor potential (TRP) cation channels function as environmental sensors primarily through Ca^{2+} signaling that can be activated by temperature, pH and osmolarity (Huang et al., 2024). The TRP channel family contains ～30 members, which can be divided into at least seven subfamilies (TRPA, TRPC, TRPM, TRPML, TRPN, TRPP and TRPV). Thermo-TRPs contain nine members from the TRPV (TRPV1-4), TRPM (TRPM2, M4, M5 and M8) and TRPA (TRPA1) subfamilies. TRPV1 and TRPV2 are activated by temperatures above 43 and 52 °C, respectively. Currents through TRPV3 exponentially increase at temperatures above 35 °C. TRPV4 can be activated by warm temperatures (27-35 °C) (Goikoetxea et al., 2021; Güler et al., 2002; Fujita et al., 2017), which are physiologically relevant to the spawning and/or sex reversal of ricefield eels. Trpv4 is abundantly expressed during testicular/sperm development, and negatively regulated by estrogen (Kumar et al., 2016). Activation of Trpv4 by various endogenous/exodogenous stimuli increases Ca^{2+} influx, participating in various downstream biological events, including gonadal cell apoptosis (Guler et al., 2002; Liu et al., 2021; Luo et al., 2023; Vrenken et al., 2016; Yamamoto et al., 2024). Remarkably, Trpv4 has been recently shown to associate temperature and sex determination in TSD alligator, and pharmaceutical activation and inhibition of Trpv4 alters testis differentiation (Yatsu et al., 2015). Based on in vitro and in vivo experiments, we propose that gonadal expressing Trpv4 is a thermosensor of ricefield eel, which links the environmental temperature and the sex determination cascades.

We provided solid evidences that the pStat3 signaling is downstream and mediates Trpv4. Previous work in TSD turtle has shown that pStat3 is involved in sex determination by transcriptionally activating the female pathway genes such as *foxl2*

and/or repressing the male pathways genes such as *kdm6b* (Chen et al., 2024; Deveson et al., 2017; Ge et al., 2018; Holleley et al., 2015; Weber et al., 2020; Wu et al., 2024). The function of *jmjd3/kdm6b* in sex determination in embryonic TISR/TSD animals has been well established (Chen et al., 2024; Ge et al., 2018; Yao et al., 2023). Overexpression and knockdown of *kdm6b* cause male to female or female to male sex reversal, respectively. In this work, increased expression of testicular differentiation genes preceded the down-regulation of ovarian differentiation genes, suggesting that male genes were directly regulated during sex change of ricefield eel. Our ChIP experiments showed that there was an enrichment of pStat3 at the promoters of *kdm6b*, suggesting that pStat3 is directly targeting this gene. Whether pStat3 regulates female genes such as *foxl2* awaits further investigation. In turtles, pStat3 functions as a repressor of *kdm6b*, whereas in ricefield eel, pStat3 functions as an activator of *kdm6b*. We propose that a yet-unidentified co-factor may determine whether it is a repressor or activator.

Trpv4, pStat3 and Kdm6b were expressed in somatic cells of ovarian tissues. After warm temperature exposure, their levels were elevated over time, similar to that of the testicular differentiation genes. We propose that the magnitude and duration of temperature exposure promote sex change of ricefield eel by driving the accumulation of testicular differentiation genes in sufficient quantities (Weber et al., 2020).

To summary, we have made a few important findings. First, the ovarian cells in ricefield eels are highly responding to the warm temperature. Second, *trpv4* expressing somatic cells surrounding the immature oocytes and the interstitial cells might be key cell types that respond to the temperature cues. Third, the pStat3-Kdm6b axis is downstream and mediates temperature-evoked Trpv4 activation. Thus, our work revealed a comprehensive TISR mechanism involves signals that initiate sex reversal (temperature) and the capture (Trpv4), sensing (Trpv4-controlled calcium influx), transduction and interpretation (the Jak/Stat3 pathway) of those environmental signals into the sex determination cascades (*kdm6b/dmrt1*). This work provides new insights into the molecular mechanism underlying natural sex change of ricefield eel. In future, it is possible to obtain non-sex change or sex-change delayed new germplasm of ricefield eels, by gene editing of the key players such as *trpv4*.

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AUTHOR CONTRIBUTIONS

Z Yang and TT Luo performed experiments and generated data; YH Sun planned and designed experiments; YH Sun wrote the manuscript; all authors edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yuhua Sun (sunyh@ihb.ac.cn).

Materials availability

All antibodies and plasmids generated in this study are available from the lead contact.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Sampling and maintenance of ricefield eels

Ricefield eels were purchased from the Baishazhou market, Wuhan, China. They were temporarily maintained in the lab at 25±1 ℃ under a 14-h light/10-h dark cycle, and fed daily with commercial diet. Female fish were usually less than 40 cm in length, males were more than 50 cm in length, and intersex animals were of medium length.

Animal experiments and treatments were performed according to the Guide for Animal Care and Use Committee of the Institute of Hydrobiology, Chinese Academy of Sciences (IHB, CAS, Protocol No. 2016-018).

RNA preparation and RNA-sequencing

We have performed RNA-sequencing (RNA-seq) experiments using gonadal tissues from female, middle-late intersex, and male animals (Zhang et al., 2025). To explore the earliest events that trigger the onset of sex change of ricefield eels, gonads from female, early intersex, and middle intersex animals were isolated. Each gonad was examined by color and morphology, and in some cases histological experiments were performed to confirm the gonadal identities. Two to three gonads from each group were pooled, and total RNAs were isolated using a TRIzol reagent (ThermoScientific, USA). RNA sample quality was checked by the OD 260/280 ratio using a Nanodrop 2000. The total mRNAs were sent to the BGI (Beijing Genomics Institute) company (ShenZhen, China), where RNA-sequencing libraries were constructed and sequenced by a BGI-500 system. RNA-seq experiments were performed at least two times, with two technical repeats.

qPCR experiments

Total RNA was isolated using the Isolation Kit mRNA. *dmrt1/sox9a* were used as male-specific genes, and *cyp19a1a/foxl2* were used as female-specific genes. The primers and the related information used in this work were listed in Table 2. Real time quantitative PCR (qPCR) analysis was used to determine gene expression levels. A total of 1 μg RNA was reverse transcribed into cDNAs using the TransScript All-in-One First-Strand cDNA synthesis Supermix (Transgen Biotech, China, AT341). qPCR amplification was carried out on a Bio-Rad CFX96 Touch Real-Time PCR System (Bio-Rad, Hercules, CA, USA) in triplicate. The reaction mixture consisted of 5 μL PerfectStart™ Green qPCR SuperMix (Transgen Biotech, China), 3.6 μL ddH2O, 0.2 μL forward and reverse primers, and 1 μL cDNA. The cycling parameters used were 94 ℃ for 30 s, 94 ℃ for 5 s, 60 ℃ for 15 s, and 72 ℃ for 10 s for 40 cycles. Quantification cycle or cycle threshold values were determined using CFX Manager 3.1 (Bio-Rad, USA). Primers were 20-21 nucleotides long, with a melting temperature between 58 and 60 ℃ and a guanine-cytosine content between 50% and 60% generating an amplicon of 80-200 bp. Beta-actin was used as a reference gene. All

qPCR experiments were repeated three times, and the relative gene expression levels were calculated based on the $2^{-\Delta\Delta Ct}$ method.

siRNA knockdown experiments

To study in vivo function of Trpv4, we used siRNA to deplete the expression of the *trpv4* gene. The sequences of the ricefield eel *trpv4* gene (Accession Number: NW_018127903.1) were obtained from NCBI GenBank. The siRNA sequences are list in Table S2. These siRNAs were purchased from Sangon Biotech (Shanghai, China). Eighteen female fish were equally divided into 3 groups: 25 ℃+ MOCK; 34 ℃+ MOCK; 34 ℃+ *trpv4*-siRNA. For the 34 ℃-group setting, after 1-2 day of acclimation at 25℃, the temperature of water was gradually increased by 3℃ per day until reaching to 34℃. For RNAi experiment, before increasing the temperatures, the siRNA was injected into ovaries through genital papilla. siRNAs were injected twice, for an interval of 2 days. Three *trpv4*-siRNAs were mixed in equal amounts; each fish was injected with 100 nM/kg. The MOCK groups were injected with equal amounts of control siRNAs. Two days after the second siRNA injection, ovarian samples were processed for qPCR analysis, and/or cryopreserved for ISH.

WB analysis

WB was performed as previously described (Sun et al., 2020; 2023). The antibodies used in this work were: Amh (Huabio, #HA500137, China), Dmrt1 (home-made), Sox9a (home-made), Foxl2 (Thermofisher, #PA1-802, USA), Stat3 (Cell signaling, #9139, USA), pStat3 (Cell Signaling, #9145, USA) (Weber et a., 2020). For animal experiments, the fish were reared for additional 3-5 days for the detection of protein of interest..

IF experiments

Anesthetic fish were fixed with 4% PFA, and gonads were isolated. The gonads were washed three times with PBS and dehydrated in sucrose solution (15% sucrose/PBS, 30% sucrose/PBS) for 2 h at 4 ℃. Gonads were mounted in Tissue-Tek OCT compound (#4583, Sakura) and sectioned to 30 μm thickness on a cryostat. Slides containing sections were dried at room temperature for 30 min and washed 3 times for 5 min at room temperature with PBS. Sections on slides were blocked using 5% normal bovine serum (#A2153, Sigma Life Science) in PBS + 0.1% Triton-X100 (#V900502, VETEC) for 2 h. After wash, Primary antibodies, including Amh (Huabio, China), Dmrt1 (home-made), Sox9a (home-made), were added at dilution of 1: 1000.

Slides were washed 3 times with PBS for 5 min, followed by incubation in Alexa 488 or Alexa 555 (#A11008/A21428, Thermofisher, USA) secondary antibodies (1: 500) for 2 h. The samples were counter-stained with DAPI (#D9542, Sigma, 1:1000) in 1 × PBS at room temperature for 1 h. After 3 times washing withPBS, slides were mountedusing an anti-fademounting medium (#HY-K1042, MedChemExpress, China). Mounted slides were imaged with a Leica Confocal Microscope (TCS SP8 STED, Germany)

In situ hybridization (ISH)

To detect the expression of *trpv4/kdm6b* in the gonads, ISH experiments were performed. The cDNAs of ricefield eel *trpv4* and *kdm6b* were amplified by gene specific primers *trpv4*-SP6-F: ATTTAGGTGACACTATAGAAGCGTTTCTAGCCATTTCCTATCGT, and *trpv4*-T7-R: TAATACGACTCACTATAGGGAGACATTATCTGCTCCTAATCGAACC. All other primers can be found in Table S1. Digoxin labeled RNA probes of Sp6-sense and T7-antisense were synthesized using the DIG RNA Labeling Kit Sp6/T7 (Roche, Basel, Switzerland). ISH was conducted following the methodology outlined by Feng et al (2011). Briefly, the fixed gonads were processed by dehydration, paraffin embedding and serial sectioning (5 μm). Then the gonad slices were digested at 37 ℃ with 200 ng/mL proteinase K for 5 min. Hybridization was carried out for 16 h at 60 ℃ using a probe concentration of 1 ng/μL in the hybridization buffer. The samples were incubated with the Anti-Digoxigenin-AP conjugate (Roche, Basel, Switzerland) at a 1: 2500 dilution for 16h at 4 ℃, and stained in NBT/BCIP staining solution (Roche, Basel, Switzerland) in the dark for 0.5–1.5h at room temperature. The results were observed and photographed using an optical microscope (Zeiss, Oberkochen, Germany). Drawings and final panels were designed using Adobe Photoshop CS6 (San Jose, CA, USA).

ChIP experiments

ChIP experiments were performed according to the Agilent Mammalian ChIP-on-chip manual. Briefly, gonadal tissues were processed into single cells and were fixed with 1% formaldehyde for 10 min at room temperature. The reactions were stopped by 0.125 M Glycine for 5 min with rotating. The fixed chromatin was sonicated to an average of (500 – 1000) bp (for ChIP qPCR) using the S2 Covaris Sonication System

(USA) according to the manual. Then Triton X-100 was added to the sonicated chromatin solutions to a final concentration of 0.1%. After centrifugation, 50 μL of supernatants were saved as input. The remainder of the chromatin solution was incubated with Dynabeads previously coupled with 5 μg ChIP grade pStat3 antibodies overnight at 4 ℃ with rotation. The next day, after 7 times washing with the wash buffer, the complexes were reverse cross-linked overnight at 65 ℃. DNAs were extracted by hydroxybenzene－chloroform－isoamyl alcohol and purified by a Phase Lock Gel (Tiangen, China). The ChIPed DNAs were dissolved in 100 μL distilled water. Quantitative real-time PCR (qRT-PCR) was performed using a Bio-Rad instrument. The enrichment was calculated relative to the amount of input as described. All experiments were repeated at least two times. The relative gene expression levels were calculated based on the $2^{-\Delta\Delta Ct}$ method. The paired t-test was used for the statistical analysis. Data were shown as means± SD.

Gonadal type identification

The gonadal types were initially identified according to morphological features including the size, shape and color. The gonadal sex of each fish was confirmed by histological sectioning and microscopic observation. In some cases, gene expression analysis was also used to confirm the gonadal types. Male genes such as *dmrt1* were not expressed in ovaries, slightly up-regulated in early ovotestes, and abundantly expressed in middle- and late-ovotestes.

Long term temperature experiments

The aim of this experiment was to assess the gonadal phenotypes of female fish that were reared at 25 ℃ (low temperature, LT) vs 33 ℃ (high temperature, HT) over 3-6 months. The fish from the Baishazhou market were transiently maintained for 3-5 day at the laboratory, and unhealthy animals were discarded. A total of approximately 400 healthy female fish (similar in body length, less than 35 cm) were randomly divided into the LT and HT groups, stocked in 10 tanks, at a density of 20 fish per tank. Fish were fed with commercial diet.

1, 3, 6 months later, one tank in each group was randomly selected, and fish were anaesthetized and measured for body length and weight. The gonads were isolated and subjected to the histological analysis to determine the gonadal types.

Short term temperature experiments

The experiments were used to explore how warm temperature treatment affects the expression of sex differentiation genes in ovaries in a short period of time. The ricefield eels were reared at 25 ℃ (lower temperature), and at 33 ℃ (warm temperature). The temperature-increase protocol started at 25 ℃, with a progressive increase of 3 ℃ per day until reaching 33 ℃. Temperature was monitored twice a day throughout the experiment. Fish were fed with Artemia daily.

Hematoxylin and eosin (H&E) experiments

H&E experiments were used for the identification of gonadal types in ricefield eels. The gonads were fixed in Bouin's solution for at least 24 h, and the H&E experiments were performed by Wuhan Company. Briefly, dehydration and paraffin imbedding were then performed on the ASP6025S Automatic Vacuum Tissue Processor (Leica, Wetzlar, Germany). The samples were sectioned using the Leica microtome (Leica) at a thickness of 5 μm. After deparaffinization, hydration and staining, the sections were examined on the Nikon ECLIPSE Ni-U microscope and micrographs were taken with the Digit Sight DS-Fi2 digital camera (Nikon).

Small molecule treatment experiments

Animal experiments

The ricefield eels from the Baishazhou market were transiently raised for 1-2 day in lower temperature conditions (25/26 ℃). The fish were then divided into 4 groups: 25 ℃+ DMSO; 25 ℃+ GSK1016790A; 34 ℃+ DMSO; 34 ℃+ RN1734. For the 34 ℃ group setting, the temperature of water was gradually increased by 3 ℃ every day until reaching to 34 ℃. For pharmaceutical experiments, before increasing the temperatures, the small molecules of appropriate concentrations were injected into ovaries. The injected dose of small molecules were optimized, and the final concentrations used were at 0.02 mg/kg body weight for RN1734, 0.01 mg/kg body weight for HO-3867 or GSK1016790A or Colivelin, and a similar volume of 1‰ DMSO were injected and served as control.

Ovarian explant or cell culture experiments

The ovaries were isolated from female ricefields and washed with cold 2% pen/strep PBS 3 times. The ovaries were cut into 2 mm3 pieces, and 0.25% TrypE were digested for 30 min, digested into single cells, after filtration, cells were cultured in 12 well plates.

For pharmaceutical experiments for Trpv4, the cells were then divided into 4 groups: 26 ℃+ DMSO; 26 ℃+ GSK1016790A; 34 ℃+ DMSO; 34 ℃+ RN1734. For the 34 ℃ group setting, after 1-2 day of acclimation at 25 ℃, the temperature of water was gradually increased by 3 ℃ every day until reaching to 34 ℃. For 34 ℃ conditions, before increasing the temperatures, the small molecules of appropriate concentrations were injected into ovaries. The injected dose of small molecules were optimized, and the final concentrations used were at 10 μM for RN1734, 100 nM for GSK1016790A and a similar volume of 1‰ DMSO were added and served as control. For pharmaceutical experiments for pStat3, similar experiments were performed, except the dose of 2 μM for HO3867 or 20 μM for Colivelin.

To determine the relationship between Trpv4 and pStat3, four culturing groups were set up: 26 ℃+ DMSO; 26 ℃+ GSK1016790A; 26 ℃+ GSK1016790A+ HO3867; 34 ℃+ DMSO; 34 ℃+ RN1734; 34 ℃+ RN1734+ Colivelin. For 34 ℃ conditions, before increasing the temperatures, the small molecules of appropriate concentrations were added into the medium.

Statistic analysis

For gene expression analyses, differences in mean values between two groups were assessed using Student's t-test. A one-way ANOVA was used to compare the expression levels of each target and the differences were determined using Tukey's post hoc test. Significance was defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Data are presented as mean standard error (SEM). Statistical analyses were conducted and graphs.

Figure Legends

Figure 1. Warm temperature promotes gonadal transformation in ricefield eel. (A) The distribution of 4 geographic populations of ricefield eels in China, showing the average annual temperature of each. (B) The design of long term temperature experiments. Two temperatures were used: 25 ℃ (cool temperature), 33 ℃ (warm temperature). (C) Representative H&E staining images showing the gonad types of animals that were reared at cool and warm temperatures at the indicated time points. Bar: 200 μm. (D) Bar graph showing the ratio of intersex/female after 180 days of cool and warm temperature treatment. The experiments were repeated at least two times.

Figure 2. *trpv4* is highly responding to environmental temperatures. (A) Relative expression levels of *trpv4* in 10 different tissues in adult ricefield eels. B: brain, H: heart, E: eye, K: kidney, S: spleen, L: liver, M:muscle, O: ovary, OT: ovotestis, T: testis. (B) ISH images showing *trpv4* expression in ovaries, ovotestes, and testes. Black arrows pointing to *trpv4* expressing cells. Bar: 200 μm. (C) Relative expression levels of the indicated *trp* and sex-biased genes of in vitro cultured ovaries at cool and warm temperatures. (D) qPCR results showing the expression patterns of *trpv4* and male sex genes in repeated temperature shifting experiments of in vitro cultured ovaries. (E) qPCR results showing the expression of *trpv4* at the indicated time points of in vitro cultured ovarian explants. (F) qPCR results showing the dynamic expression of the indicated genes in gonads of female ricefield eel at 25 ℃, and at the indicated time points after shifting to 34 ℃. day 1: day 1 after shifting to 34 ℃. (G) ISH images showing the dynamic expression of *trpv4* at the indicated time points after shifting to 34 ℃. Bar: 200 μm. *: $P< 0.05$, **: $P< 0.01$, ***: $P< 0.001$, and ****: $P< 0.0001$. ns: not significant. All experiments were repeated at least three times.

Figure 3. Animal experiments show that warm temperature induced male gene expression depends on Trpv4. (A) Cartoon showing the design of experiments. Female eels kept at cool (25 ℃) and warm (34 ℃) temperatures were injected with the Trpv4 agonist GSK1016790A and antagonist RN1734 into the ovaries, respectively. After 2-3 days of injection, the ovaries were isolated and processed for the subsequent experiments. (B) Relative expression levels of the sex-biased genes at the indicated conditions. (C) Representative WB images showing the expression of the indicated markers at the indicated conditions. Relative quantification for each protein was calculated and shown in the mages. (D) Representative IF images showing the expression of male/testicular differentiation genes at the indicated conditions. Bar: 200 μm. (E) quantification of panel D. *: $P< 0.05$, **: $P< 0.01$, ***: $P< 0.001$, and ****: $P< 0.0001$. ns: not significant. All experiments were repeated at least three times.

Figure 4. siRNA-mediated *trpv4* knockdown abolishes the abnormal up-regulation of male genes by warm temperature treatment. (A) qPCR results showing the relative expression of the indicated genes at the indicated conditions. (B) Representative WB images showing the expression of sex biased proteins in the

indicated conditions. *: $P< 0.05$, **: $P< 0.01$, ***: $P< 0.001$, and ****: $P< 0.0001$. ns: not significant. All experiments were repeated at least three times.

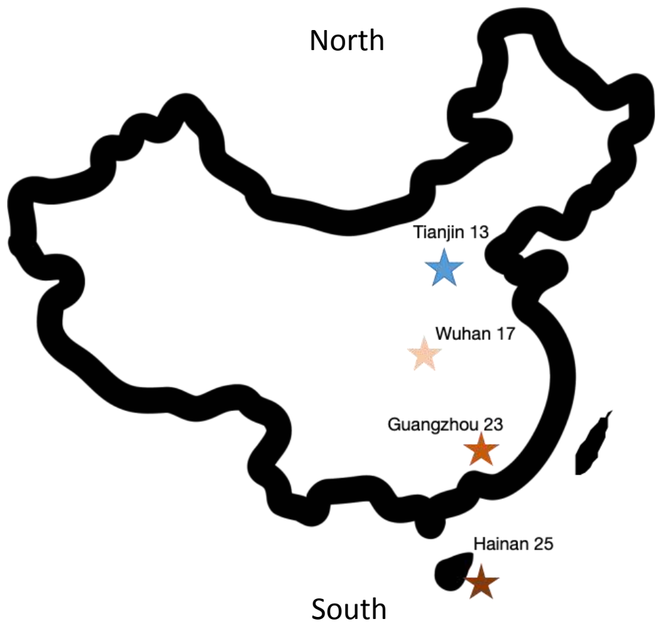
Figure 5. The JAK/Stat3 signaling is downstream of Trpv4. (A) Heat map showing the expression of the indicated genes of different groups. (B) IF images showing the expression of pStat3 in the indicated conditions. The white arrows indicated the location of pStat3 expressing cells. The experiments were repeated at least two times. Bar: 200 μm. (C) Quantification of calcium signals in the indicated conditions. Ovarian explants were cultured at the indicated conditions, and calcium signals were determined by calcium indicator dye Cal-520 acetoxymethyl ester. (D) Representative WB images showing the expression of the indicated makers in ovaries, early ovotestes, and middle ovotestes.

Figure 6. Animal experiments showing that warm temperature induced male gene expression depends on pStat3. (A) Cartoon showing the design of the experiments. Female eels kept at cool (25 ℃) and warm (34 ℃) temperatures were injected with the pStat3 agonist Colivelin and antagonist HO3867 into the ovaries, respectively. After 2-3 days of injection, the ovaries were isolated and processed for the subsequent experiments. (B) Relative expression of the indicated genes at the indicated conditions. (C) IF images showing the expression of male biased genes in the indicated conditions. Bar: 200 μm. (D) Quantification of panel C. (E) Relative expression of the indicated genes at the indicated conditions. *: $P< 0.05$, **: $P< 0.01$, ***: $P< 0.001$, and ****: $P< 0.0001$. ns: not significant. All experiments were repeated at least three times.

Figure 7. pStat3 binds and activates the *kdm6b* gene. (A) Cartoon showing the conserved pStat3 binding motifs upstream the kdm6b TSS. (B) ChIP experiments showing the enrichment of pStat3 at the *kdm6b* locus in ovarian tissues of fish reared at cool temperature (CT), and warm temperature (WT) in the absence and presence of HO-3867. (C) ISH images showing the expression of *kdm6b* in ovary and testis. Bar: 200 μm. (D) Cartoon showing the mechanism of Trpv4-mediated temperature induced sex change in ricefield eel.

Figure 1 Warm temperature promotes gonadal transformation in ricefield eel

A



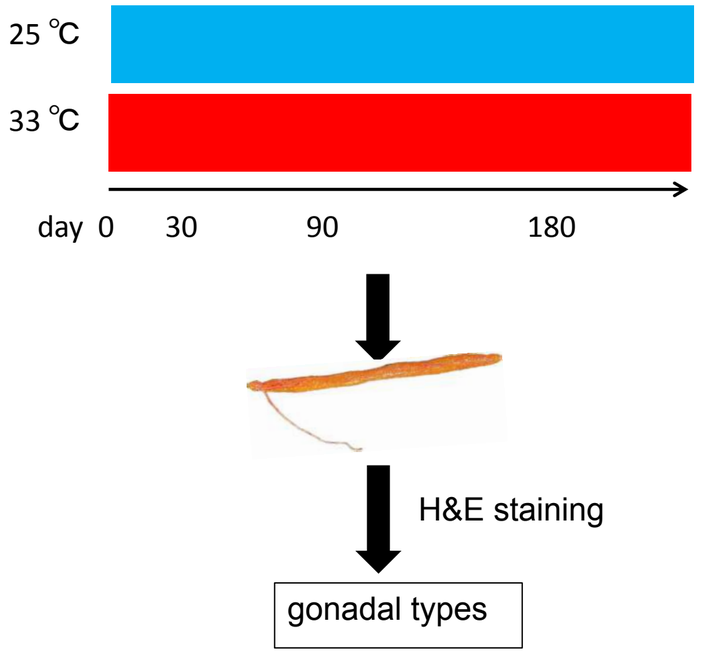
The average annual temperature of different regions from North to South:
Tianjin: 13 ℃

Wuhan: 17 ℃

Guangzhou: 23 ℃

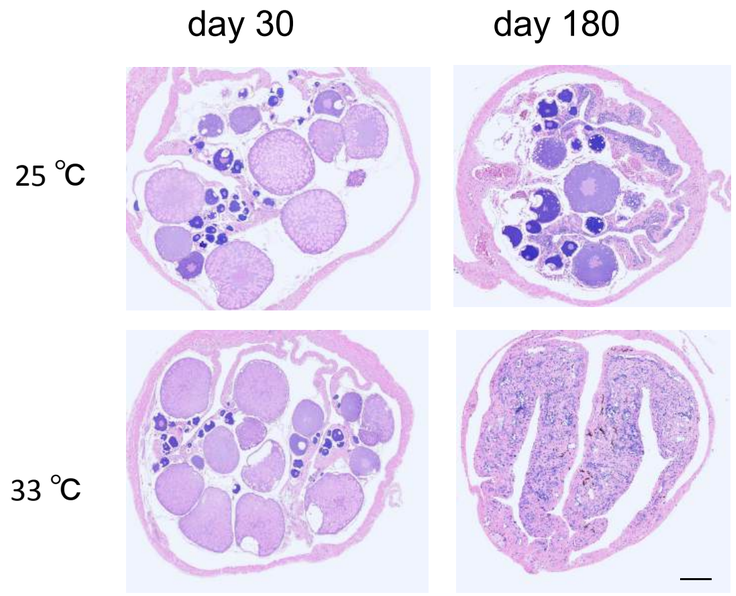
Hainan: 25 ℃

B



C

H&E staining images showing gonadal tissues at the indicated time points



D

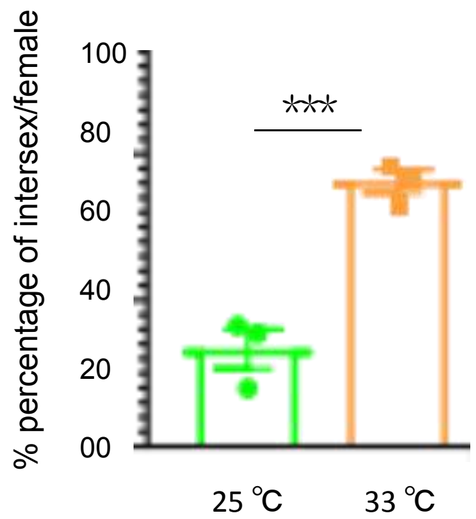
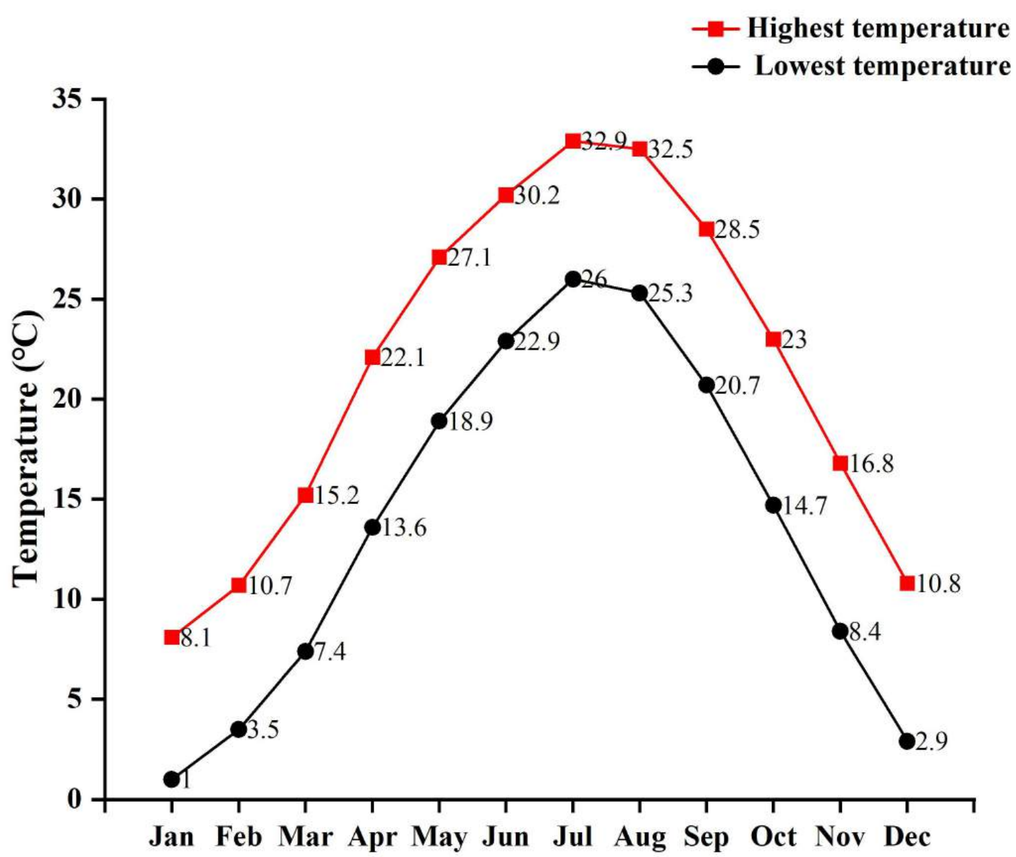


Figure S1 Warm temperature induces the sex change

A

Average temperatures for each month, 1991-2021 year



B

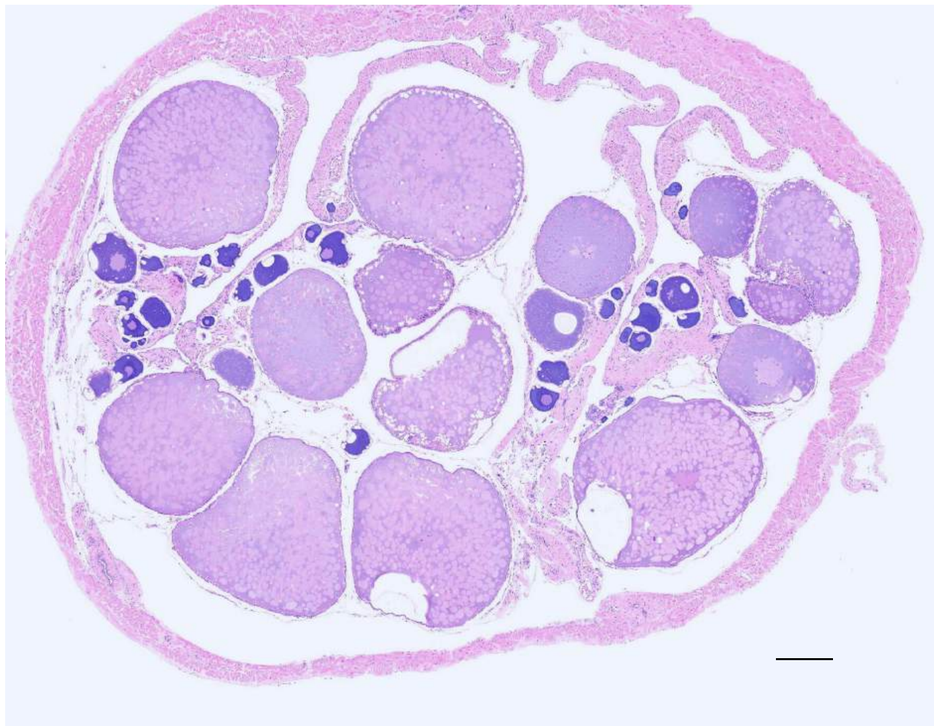


Figure 2 *trpv4* is highly responding to warm temperatures

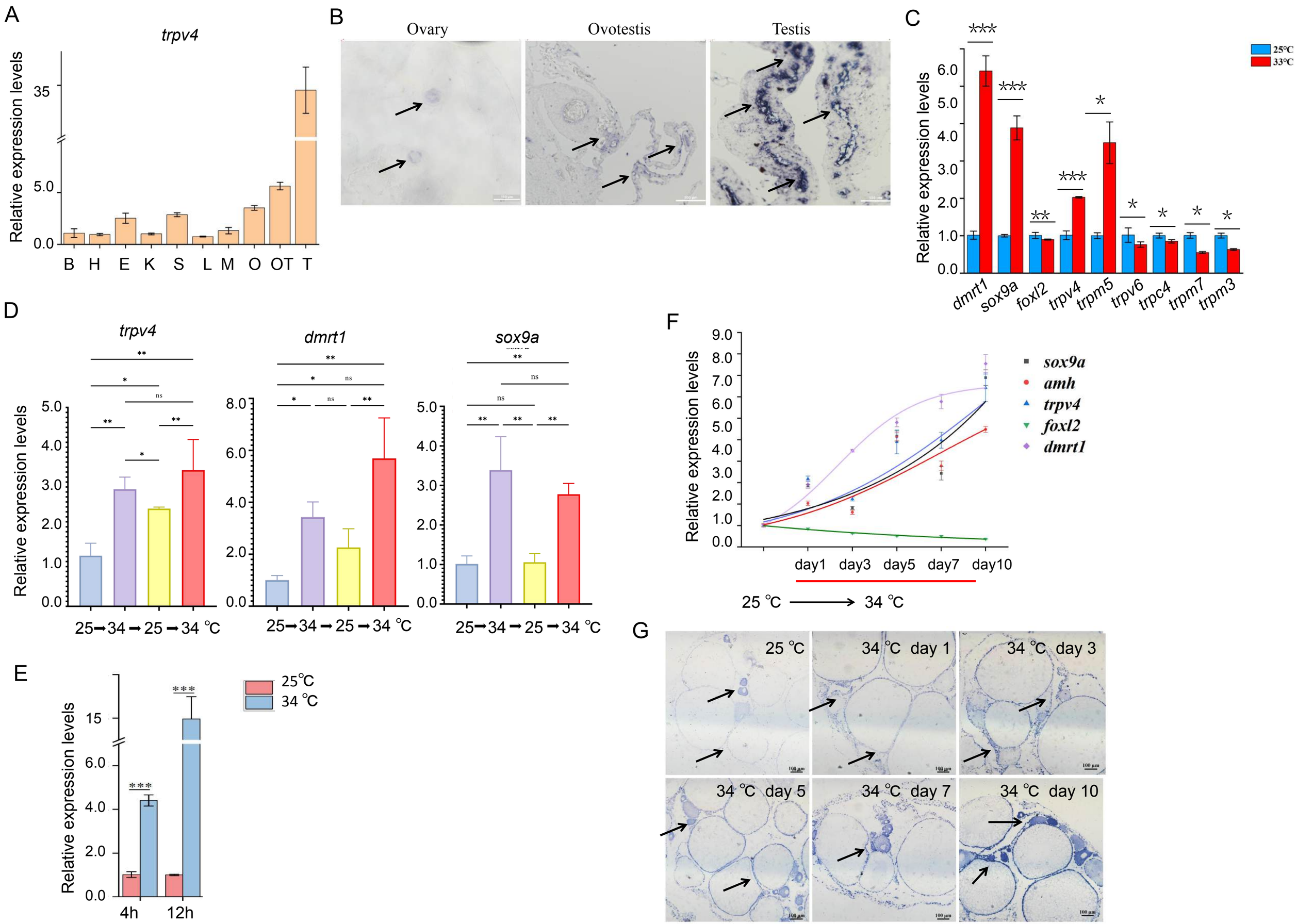
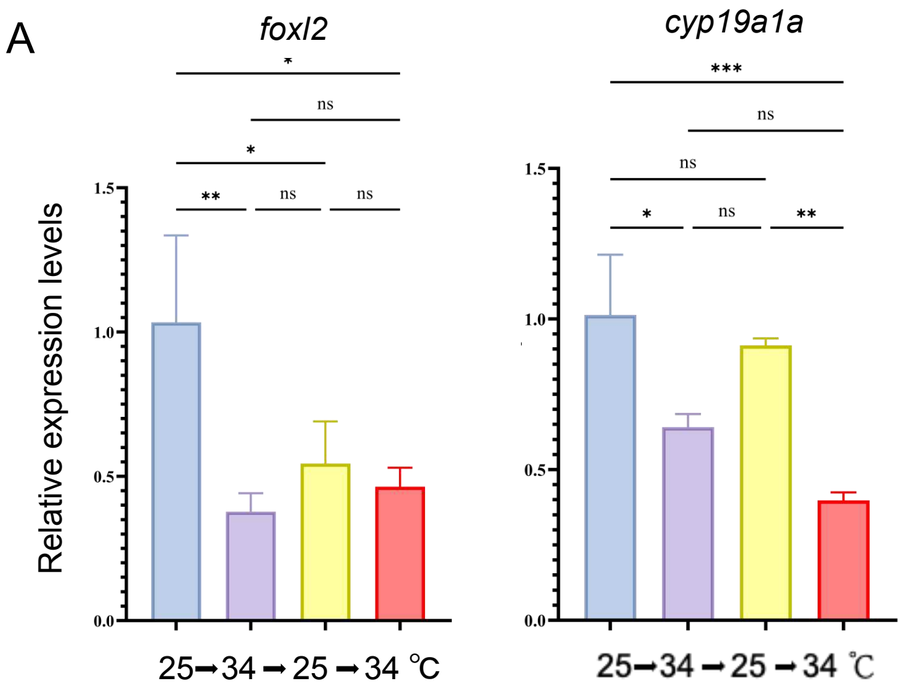
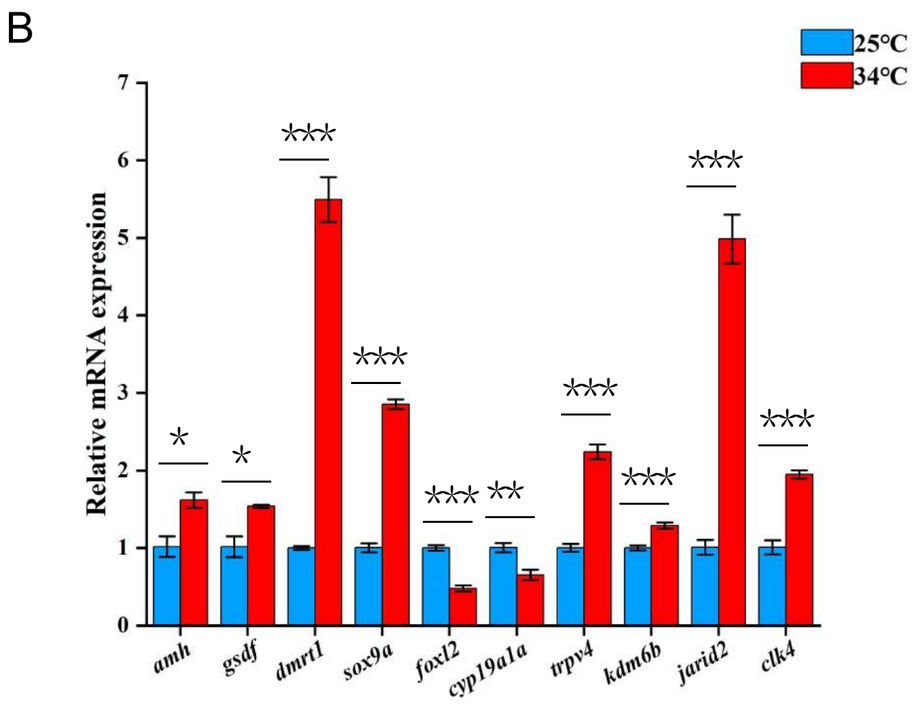


Figure S2 The expression of *trpv4* is induced by high temperatures





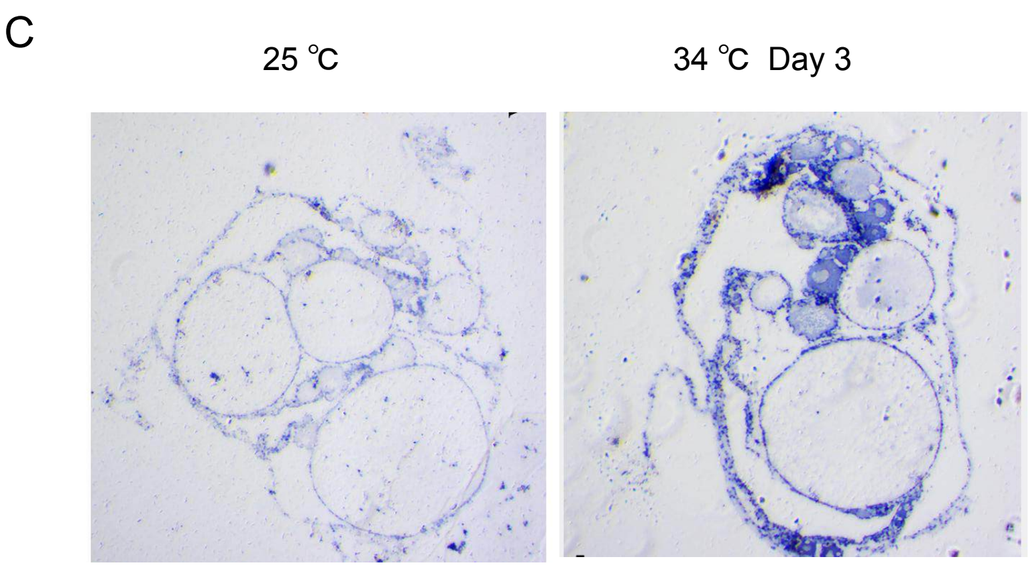
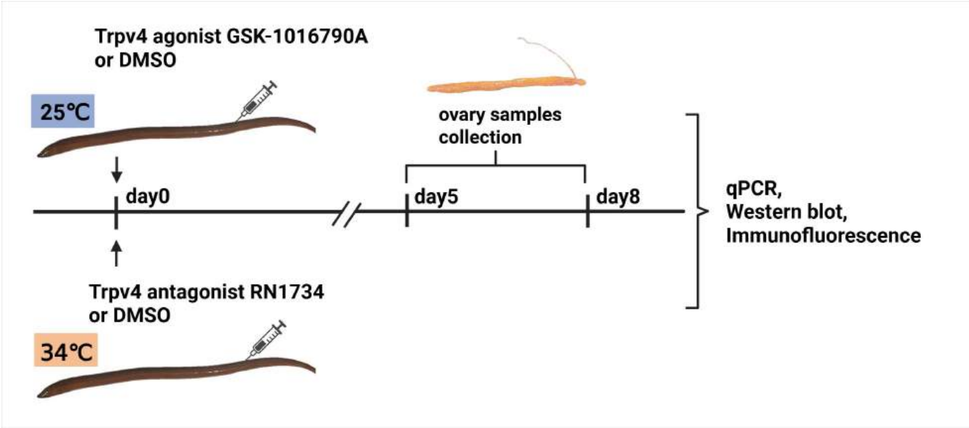


Figure 3 Warm temperature-induced male gene expression depends on Trpv4

A



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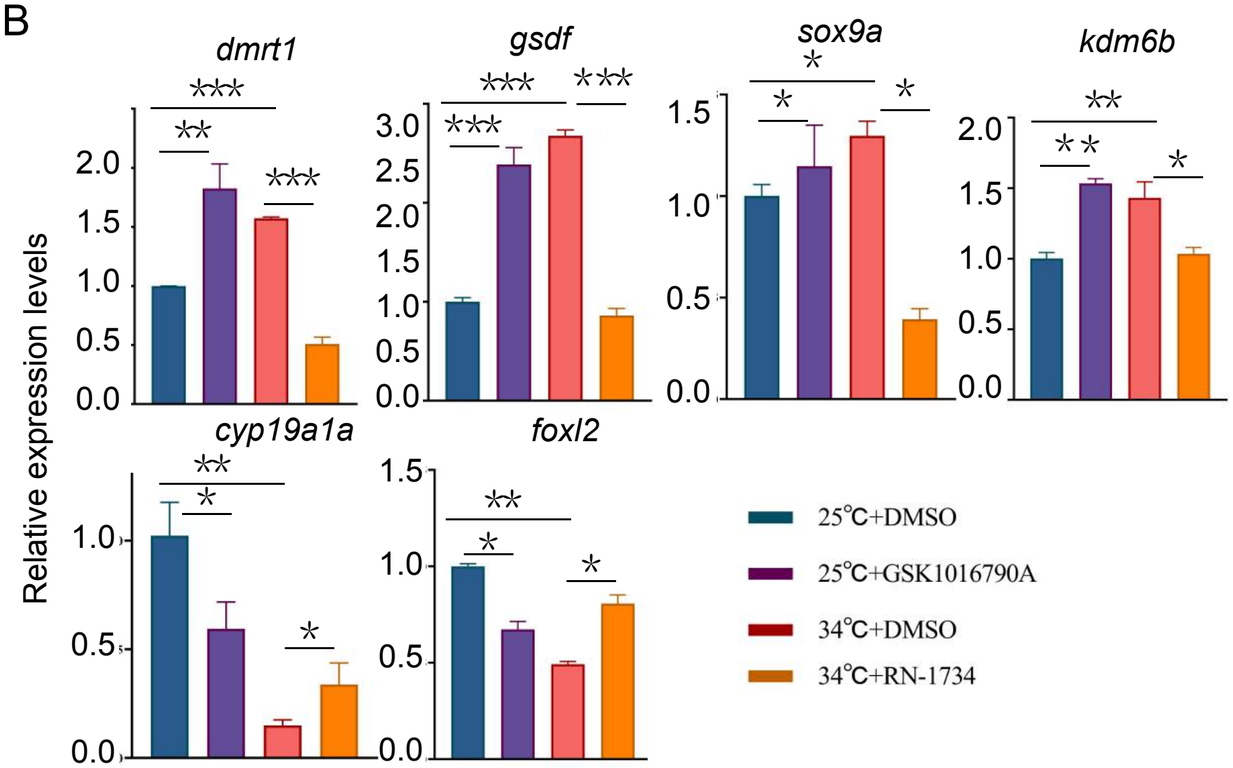

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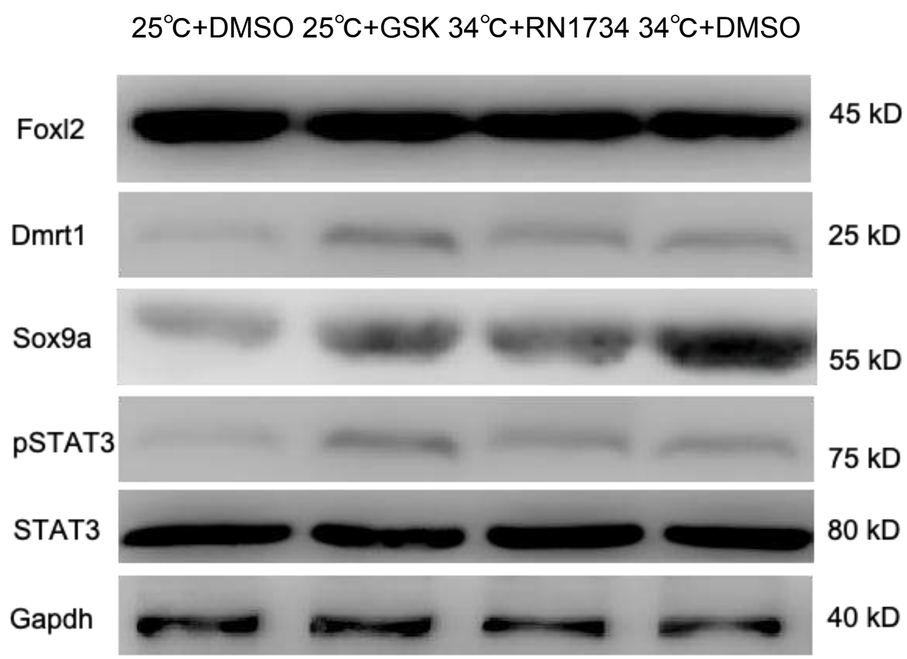

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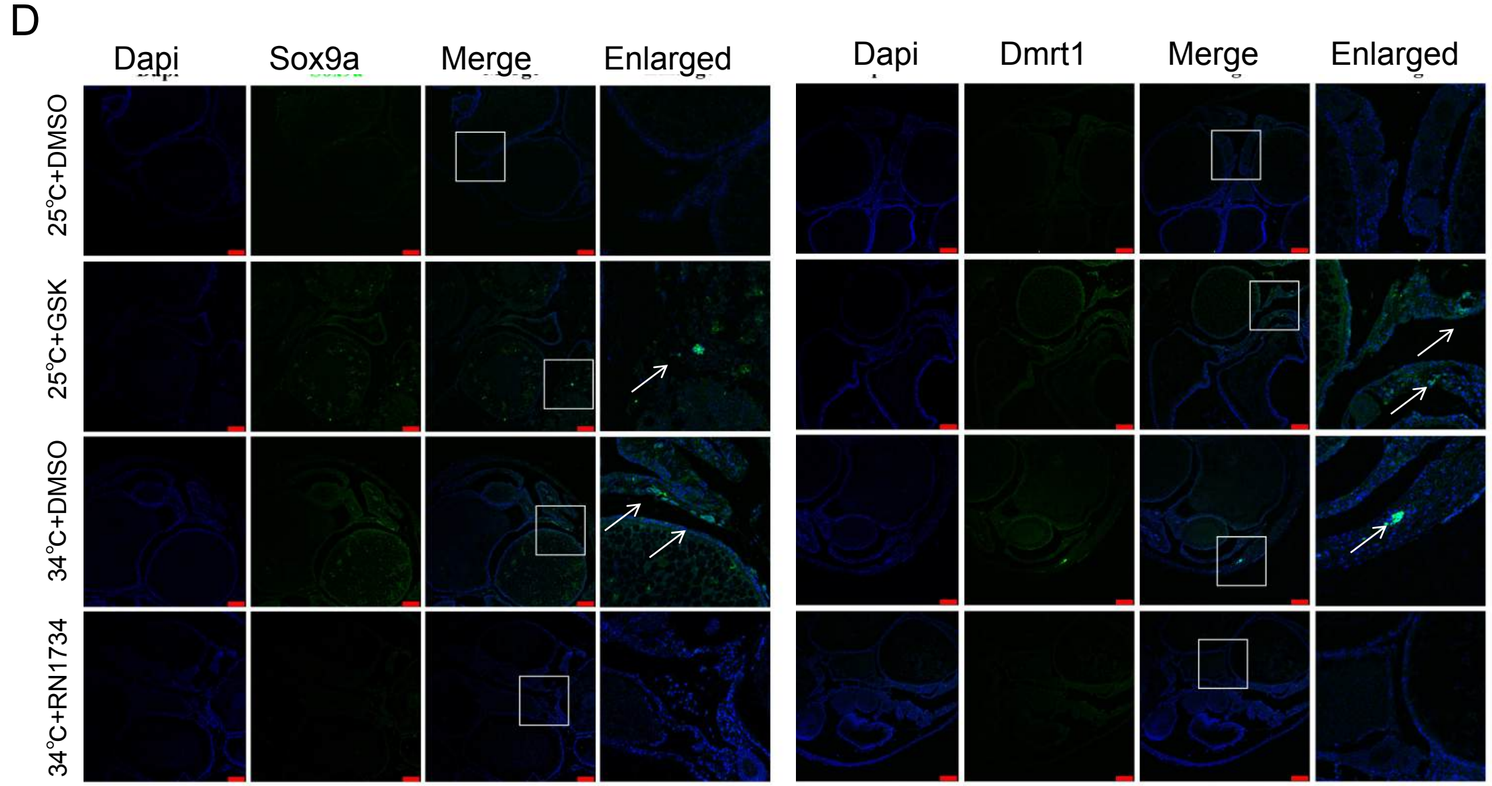

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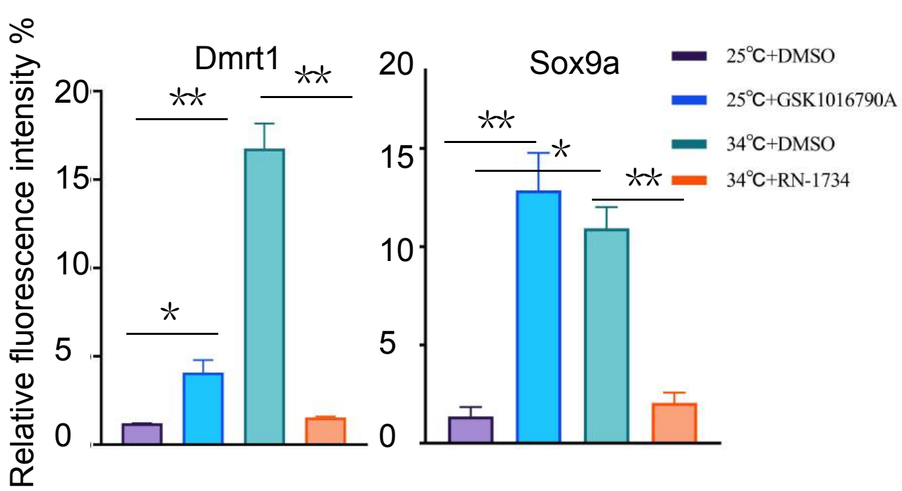
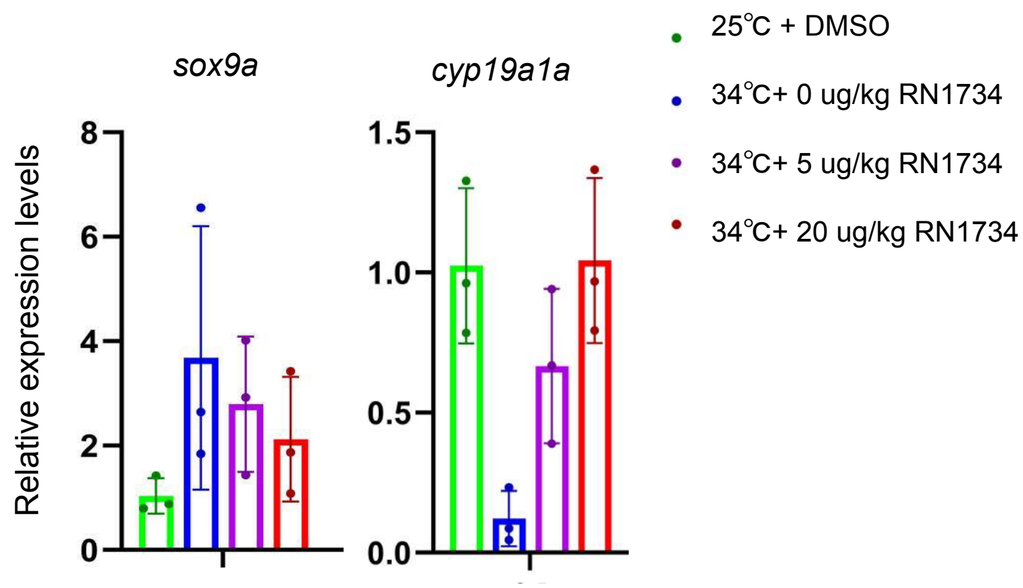


Figure S3 Temperature induced male gene expression depends on Trpv4

A



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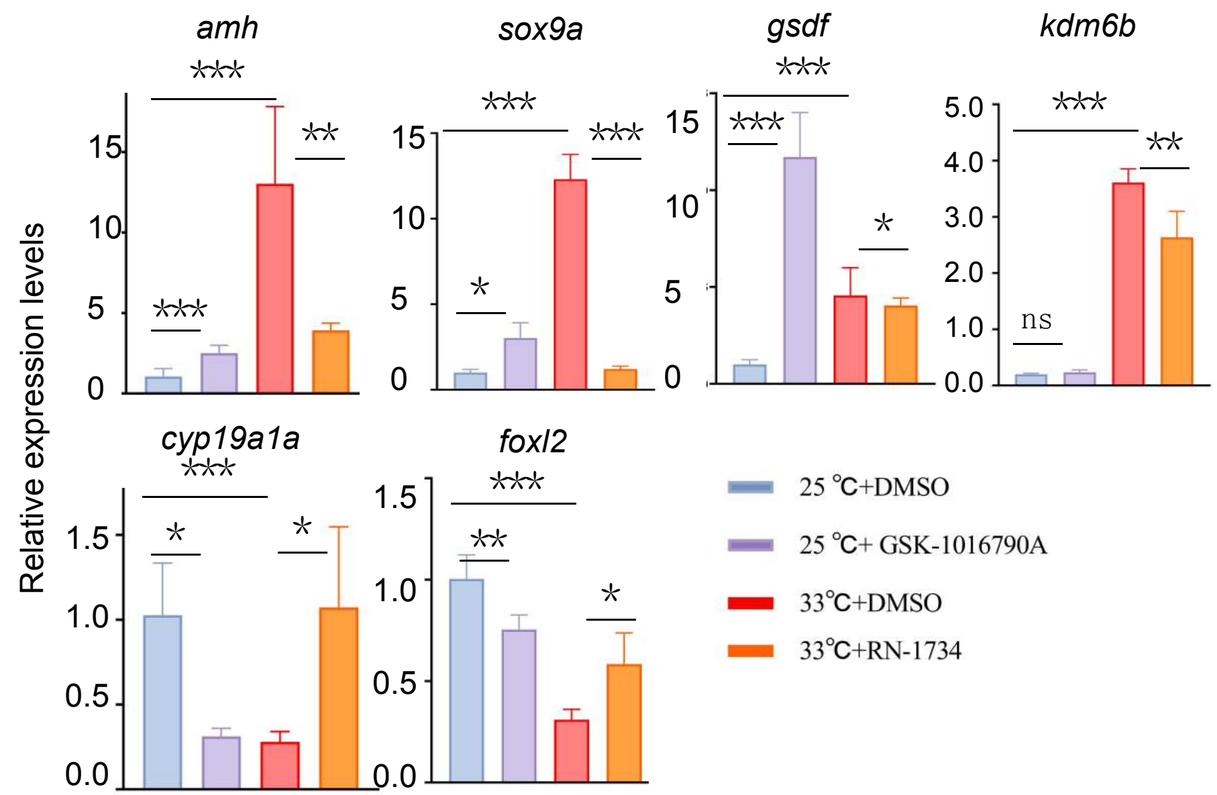

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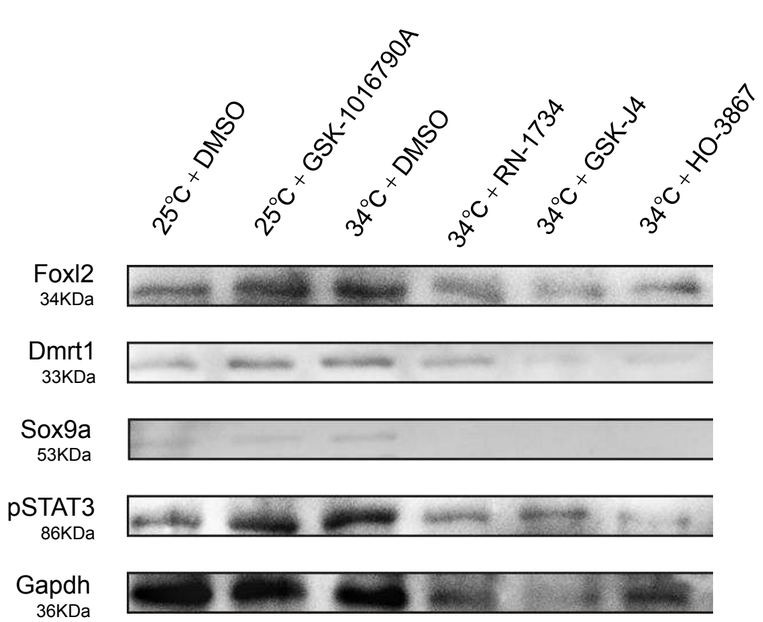

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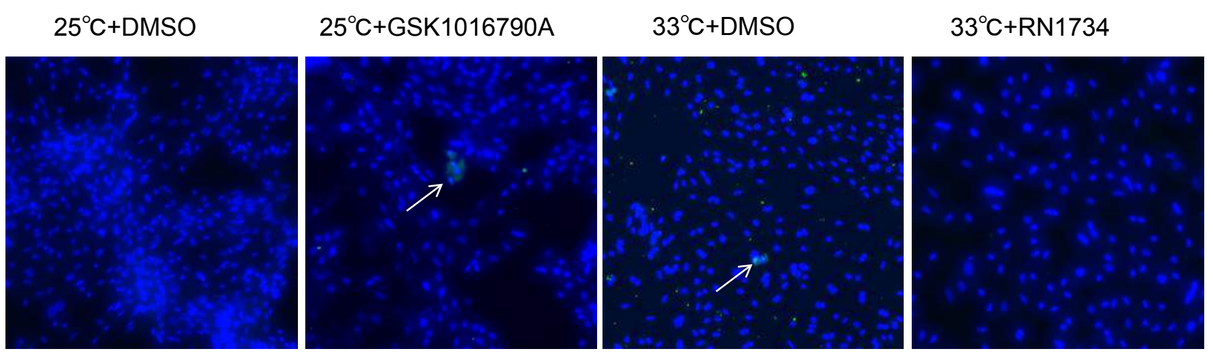


Figure 4 Trpv4 siRNA knockdown abolishes the up-regulation of male genes by warm temperature

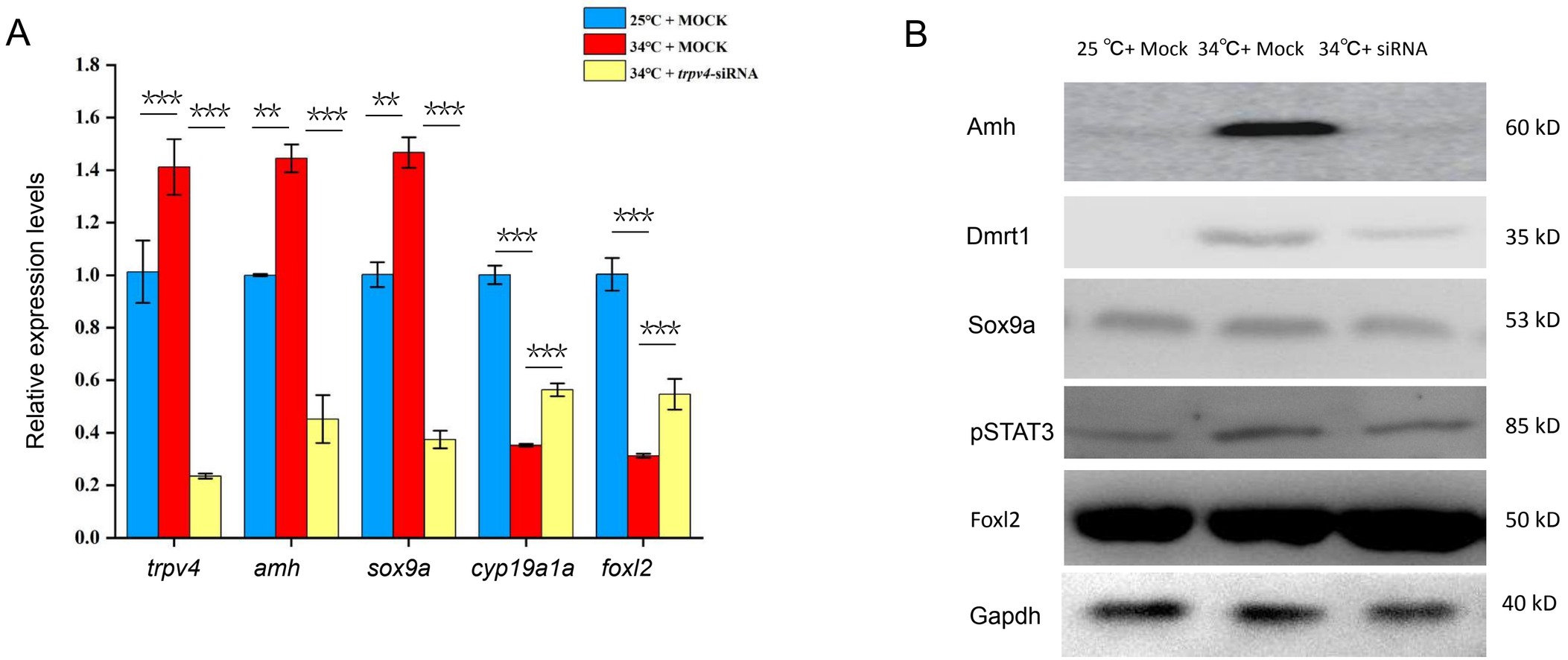


Figure 5 The pStat3 signaling is downstream of Trpv4

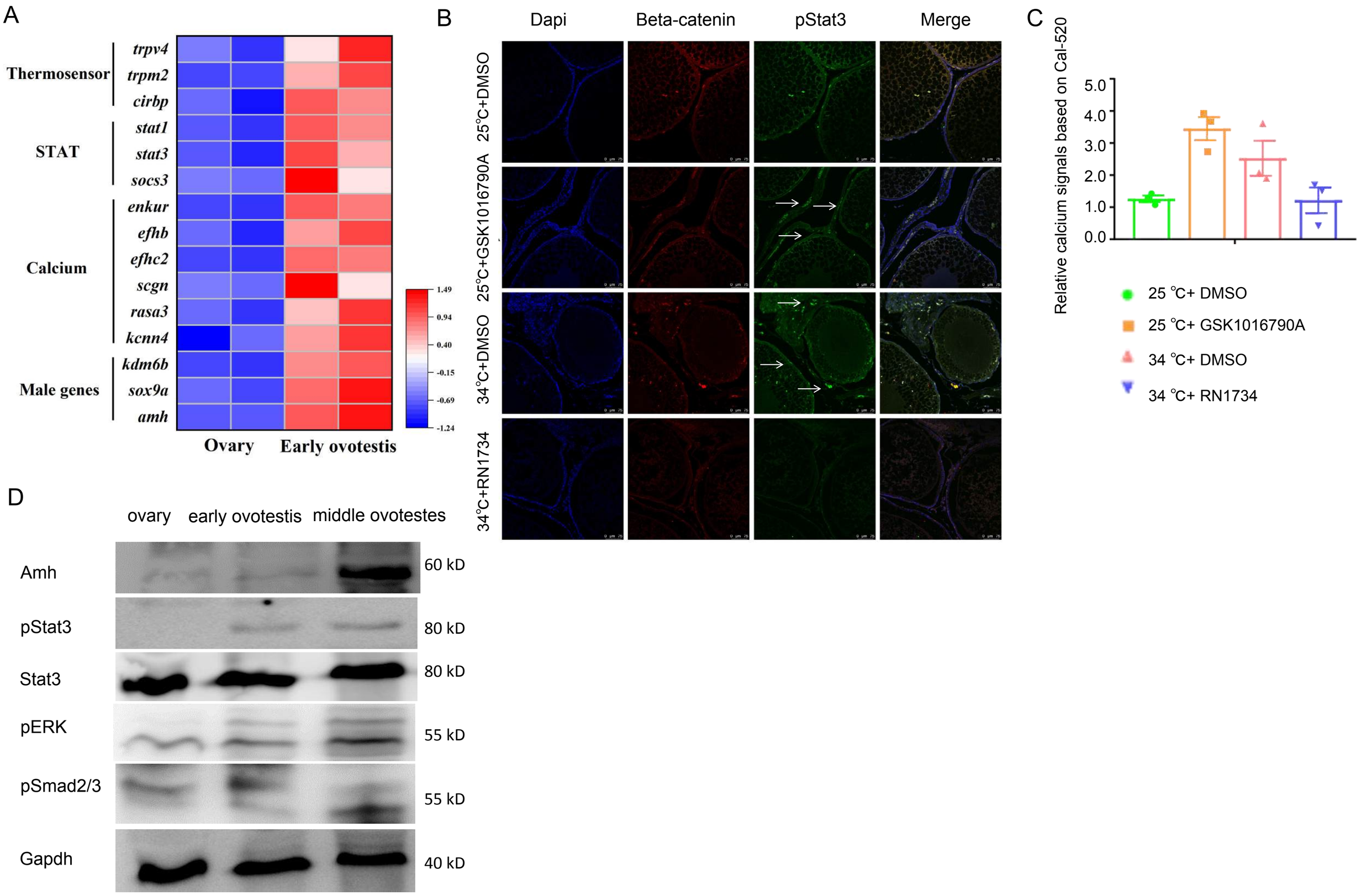
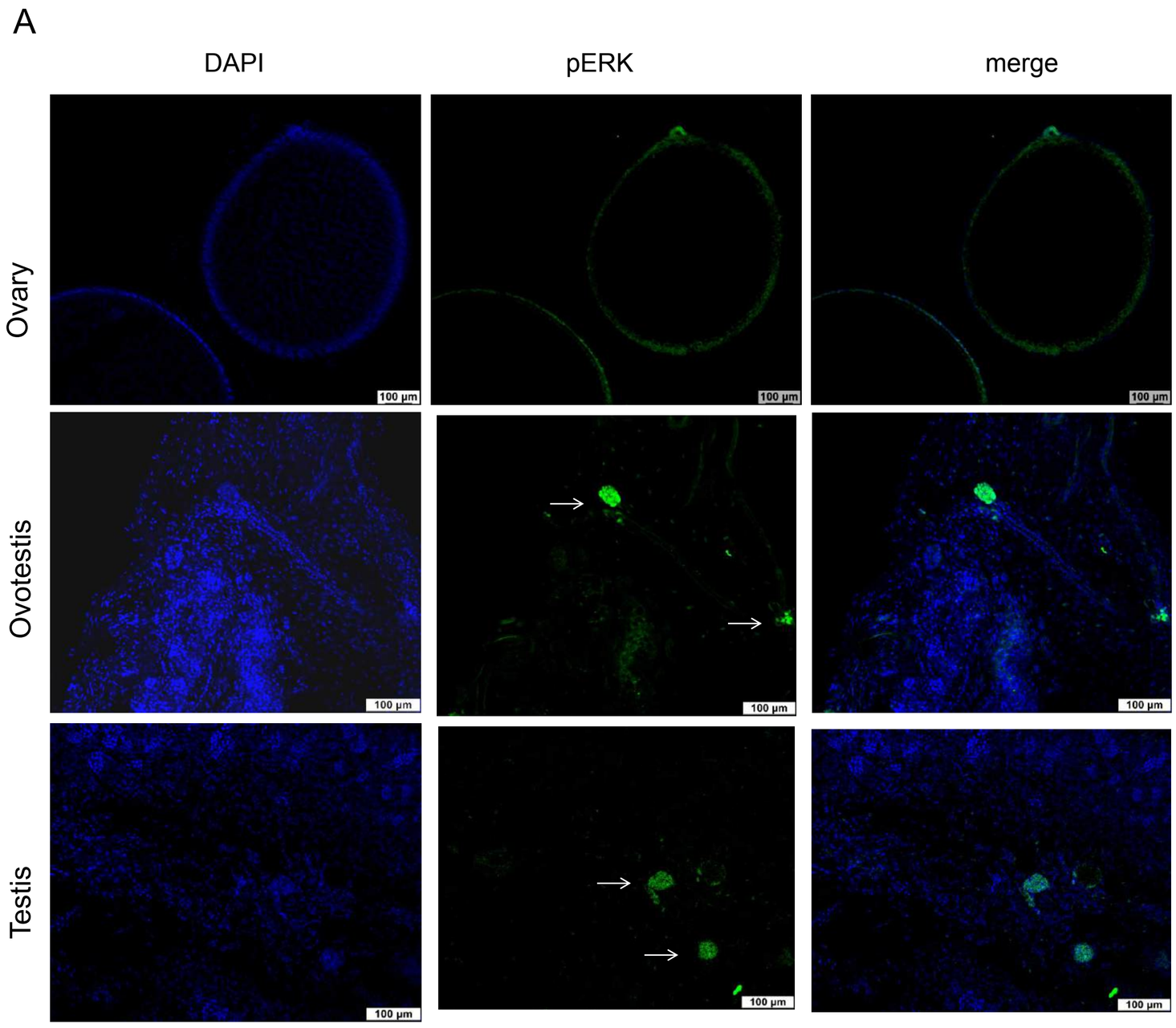


Figure S4 Related to Figure 5



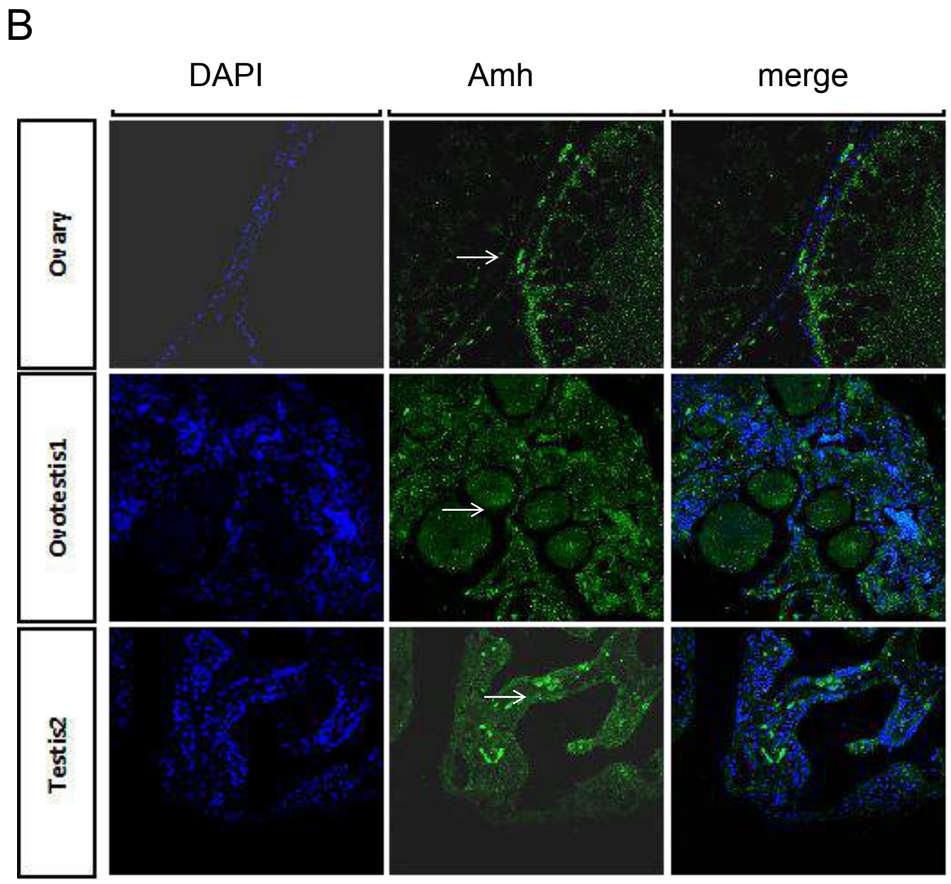


Figure 6 Temperature-induced male gene expression depends on pSTAT3

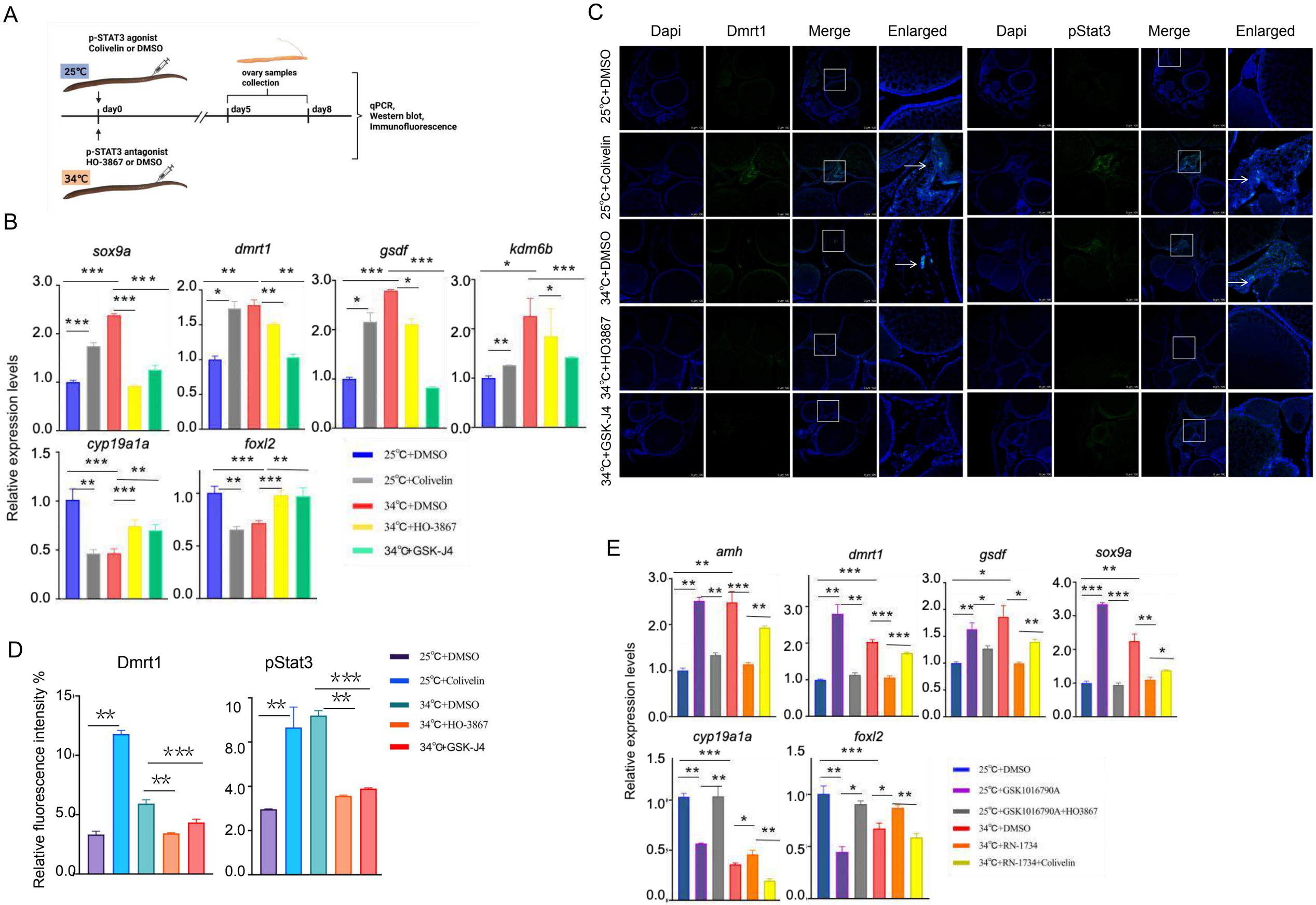
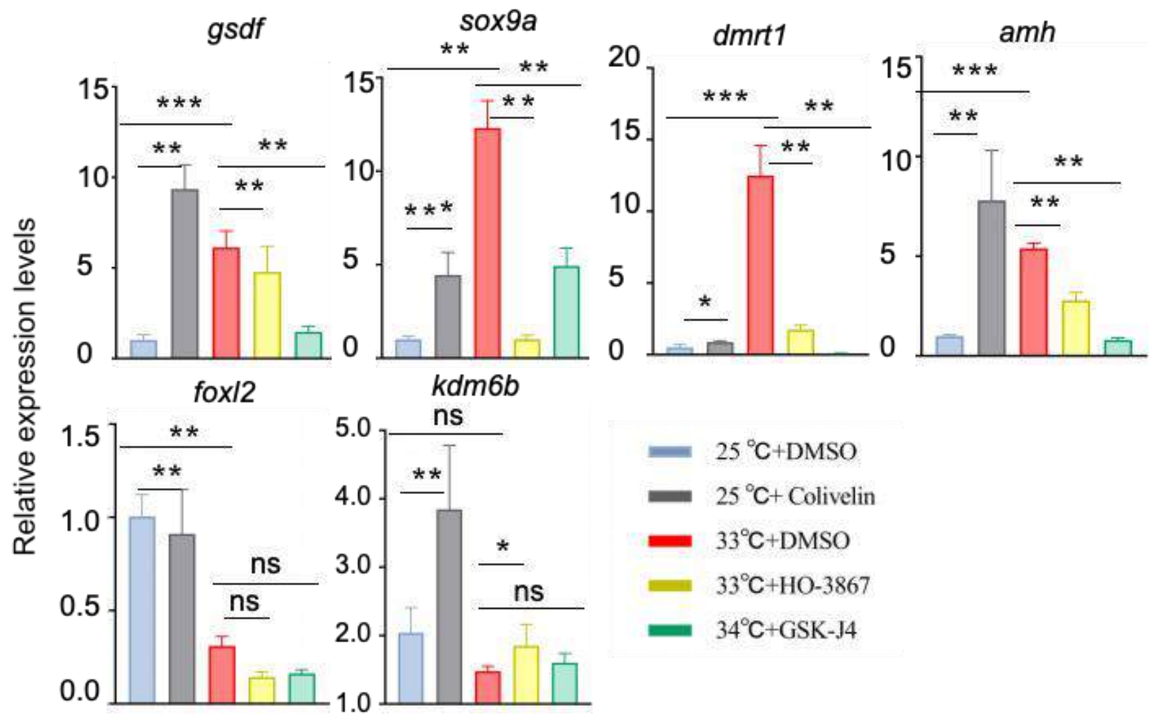


Figure S6 Temperature induced male gene expression depends on pSTAT3 (cell culture)

A



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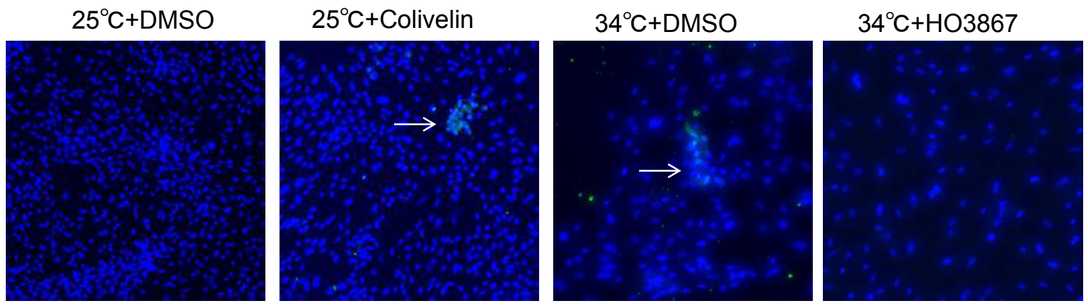

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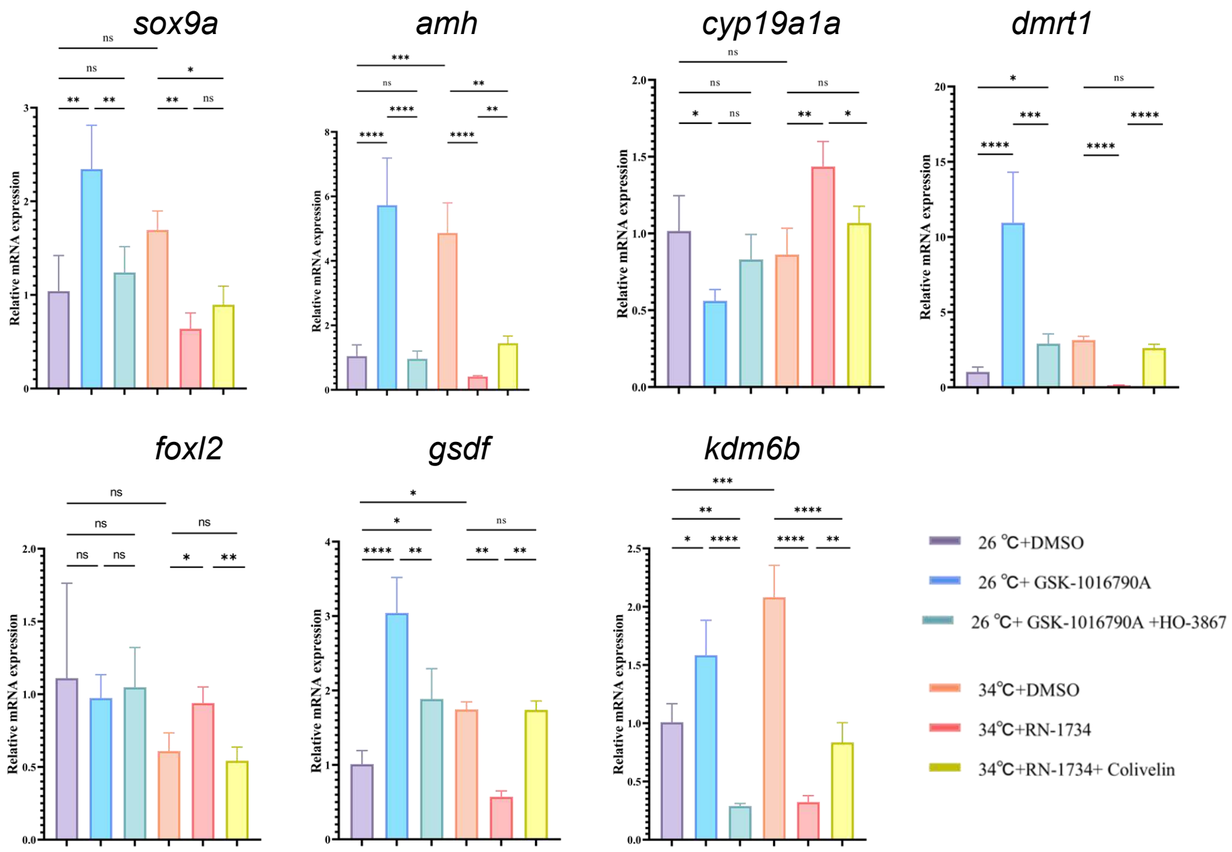
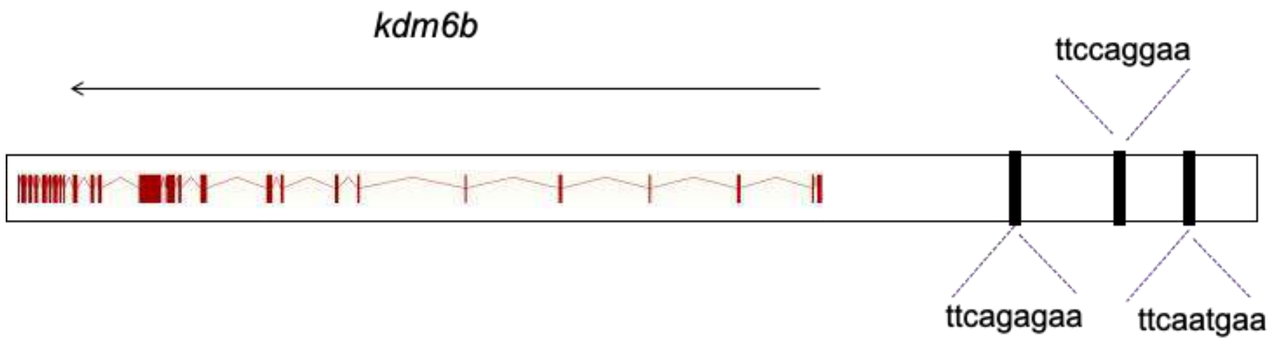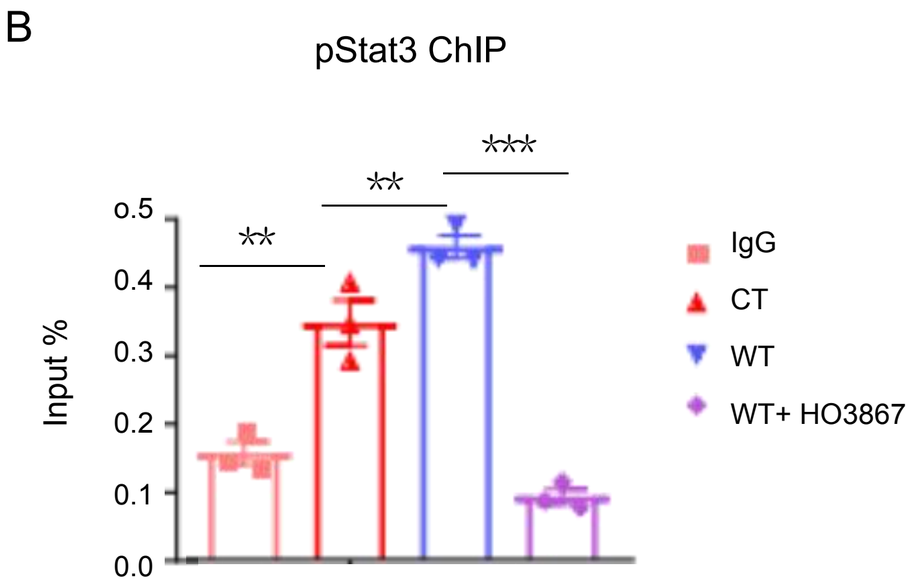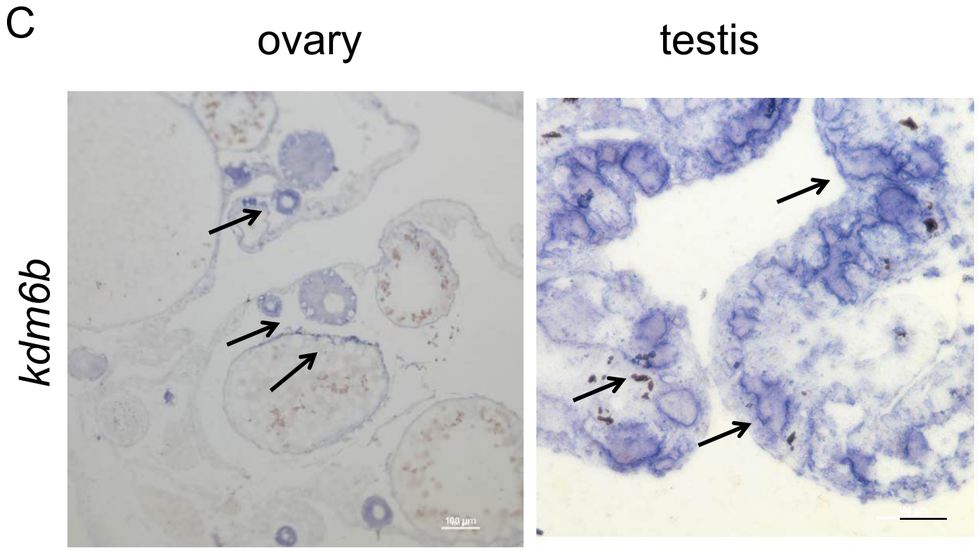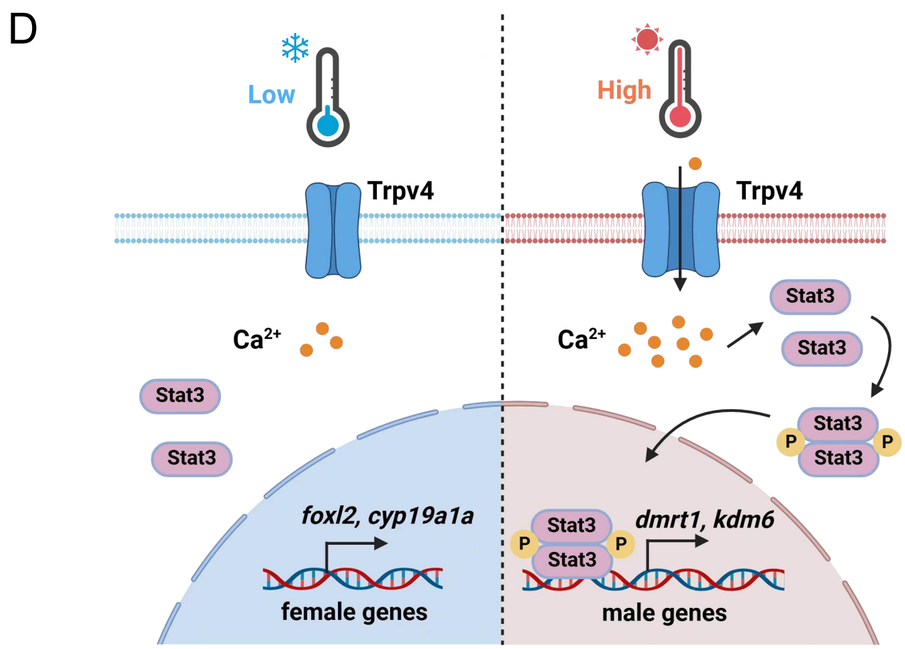
Figure 7 pSTAT3 binds and activates *kdm6b*