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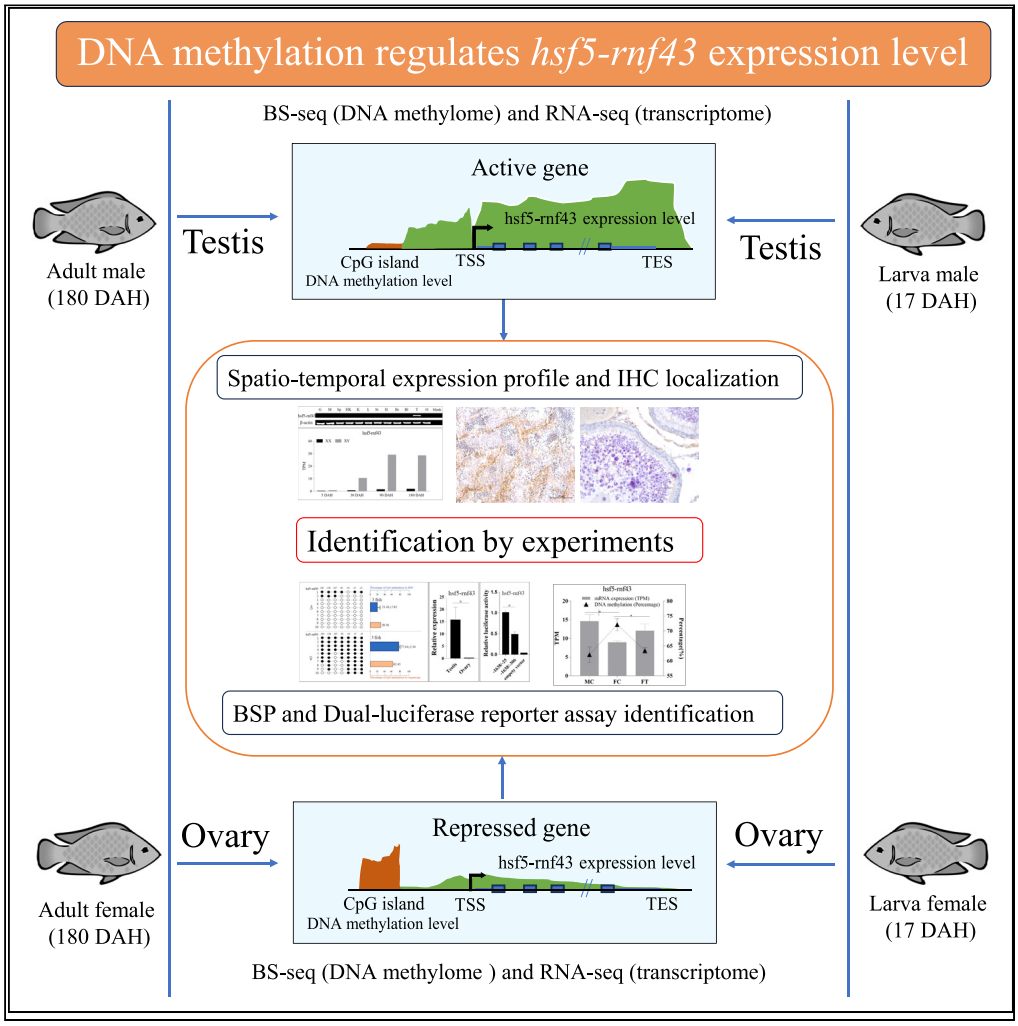

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Highlights

The genes *hsf5* and *rnf43* fused to form *hsf5-rnf43* in Nile tilapia

Hsf5-rnf43 was located in primary spermatocytes and increased as testis developed

DNA methylation controlled *hsf5-rnf43* expression as testis developed

Temperature affected DNA methylation regulating *hsf5-rnf43* sex-biased expression

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The fusion gene hsf5-rnf43 in Nile tilapia: A potential regulator in the maintenance of testis function and sexual differentiation

Yawei Shen,[1,2,6] Hewei Jiang,[2,6] Adelino V. M. Canario,[4] Tiantian Chen,[2] Yufei Liu,[2] Guokun Yang,[1] Xiaolin Meng,[1] Jinliang Zhao,[2,*] and Xiaowu Chen[3,5,*]

SUMMARY

We identified that the genes heat shock transcription factor 5 (*hsf5*) and ring finger protein 43 (*rnf43*) happened fusion in Nile tilapia (*Oreochromis niloticus*), called *hsf5-rnf43*, and provided the characteristic and functional analysis of *hsf5-rnf43* gene in fish for the first time. Analysis of spatiotemporal expression showed that *hsf5-rnf43* was specifically expressed in the testis and located in primary spermatocytes of adult Nile tilapia and gradually increased during testis development from 5 to 180 days after hatching. We also found DNA methylation regulated sex-biased expression of *hsf5-rnf43* in the early development of Nile tilapia, and was affected by high temperature during the thermosensitive period of Nile tilapia sex differentiation. Therefore, we first reported that the fusion gene *hsf5-rnf43* was sex-biased expressed in the testis regulated by DNA methylation and affected by high temperature, which may be involved in the maintenance of testis function and sex differentiation of Nile tilapia.

INTRODUCTION

Being a significant farmed fish, Nile tilapia displays a notably faster growth rate in males compared to females.[1] The preference for all-male stocks arises from their ability to enhance the efficiency of commercial production. Consequently, it is essential to explore the molecular mechanism of sex determination and maintenance in Nile tilapia. The mechanisms that sex determination and differentiation in teleosts are broadly classified as environmental sex determination (ESD), genetic sex determination (GSD), and combining ESD and GSD.[2] Another potential way to regulate sex determination is DNA methylation.[3,4] DNA methylation occurs in the genomes of a wide array of bacteria, plants, fungi, and animals. DNA methylation in vertebrates typically occurs at cytosine-phosphate-guanine sites (CpG) and is catalyzed by DNA methyltransferase.[5] DNA methylation of the CpG site in the promoter region can inhibit the expression of many genes and is an important research object in epigenetics.[6] Comparative analysis of the gonadal DNA methylation of pseudomale, female, and normal male fish revealed that genes in the sex determination pathways are the major targets of substantial methylation modification during sexual reversal in tongue sole fish.[7] The DNA methylation of some sex-related genes such as aromatase (*cyp19a*) may be an important means to regulate gene expression in mature gonadal function maintenance in tilapia through genome-wide mapping of DNA methylation in Nile Tilapia.[8]

Gene fusion is a complex process involving the fusion of complete or partial sequences from two or more distinct genes, resulting in the creation of a singular chimeric gene or transcript. Research into gene fusions sheds light on their origin mechanisms and the diverse functional changes accompanying their generation. Gene fusions prominently contribute to evolutionary change by providing a continuous source of new genes.[9] Ordinarily, gene transcription reaches a regulated termination point, which prevents the RNA polymerase from traversing into the next gene. There have been reports indicating that chimeric transcripts formed by transcribing two consecutive genes into a single RNA molecule can manifest in humans. Through the splicing and translation of such RNAs, a novel fusion protein can emerge that contains domains from both original proteins.[10]

Heat shock transcription factor 5 (Hsf5) is predominantly expressed in the testis and is essential for spermatogenesis in zebrafish[11] and mice.[12] Hsf5 mutations cause low sperm count and structural defects.[11] Hsf5 was also correlated with immune infiltration and serves as a prognostic biomarker in lung adenocarcinoma.[13] Ring finger protein 43 (Rnf43) functions as a tumor suppressor that inhibits Wnt/β-catenin signaling by ubiquitinating Frizzled receptor and targeting it to the lysosomal pathway for degradation.[14] Loss of function of Rnf43 results in proliferation and tumor growth. Mutations of Rnf43 have been reported in different cancers, such as colorectal and endometrial cancers,[15]

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pancreatic ductal adenocarcinoma,[16] gastric carcinoma,[17] serrated adenoma,[18] and lynch syndrome adenomas.[19] We have done the work to analyze the whole-genome DNA methylation and transcriptome difference between the testis and ovary in Nile tilapia by using methylated DNA immunoprecipitation sequencing (MeDIP-Seq) and RNA-seq technology.[8] We speculated that *hsf5* and *rnf43* may be fused to form new genes and play new functions. This study will explore and verify this fusion phenomenon and whether it may serve as a new mechanism regulating sex determination and maintenance in Nile tilapia.

RESULTS

Molecular characterization analysis of heat shock transcription factor 5-ring finger protein 43

The full-length sequence of *hsf5-rnf43* is 6445 base pair (bp) containing an open reading frame of 3492 bp, which encoded a putative protein of 1163 amino acids. The 5′ untranslated region (5′-UTR) was 262 bp, and the 3′-UTR was 2691 bp (Data S1). The detailed information of *hsf5-rnf43* gene sequence was submitted to the GenBank database in National Center for Biotechnology Information (GenBank: OR591159). The relative molecular mass and theoretical isoelectric point of the protein were 12.80 kDa and 6.64, respectively (Figure S1). Multiple alignments between Hsf5 and Hsf5-rnf43 amino acid sequences showed that they have conserved amino acids in the front-end sequence of Hsf5-rnf43. Multiple alignments between Rnf43 and Hsf5-rnf43 amino acid sequences showed that they have conserved amino acids in the back-end sequence of Hsf5-rnf43 (Figure S2). The putative conserved domains in Hsf5-rnf43 protein among *O. niloticus*, *O. aureus*, and *H. burtoni* both have HSF DNA-bind, ring finger 3 ectodomain (ZNRF-3 ecto), and ring finger (RING) domains. These domains are separately distributed in Hsf5 and Rnf43 protein of *D. rerio*, *Neolamprologus brichardi*, *Archocentrus centrarchus*, *Microcaecilia unicolor*, *Lacerta agilis*, *Chelonia mydas*, *Gallus gallus*, *Delphinapterus leucas*, and *Homo sapiens* (Figure S3). The cross-species comparison of the chromosomal location among *O. niloticus*, *D. rerio*, *X. tropicalis*, *L. agilis*, *C. mydas*, *G. gallus*, and *H. sapiens* indicated other species is not happed gene fusion of *hsf5* and *rnf43* except for *O. niloticus*. *Hsf5-rnf43* gene was flanked by several genes, including *myotubularin-related protein 4* (*mtmr4*), *transcription elongation factor SPT4* (*supt4h1*), and *translocator protein associated protein 1* (*tspoap1*) (Figure S4).

We only found eight species of bony fish (*O. niloticus*, *Oreochromis aureus*, *Simochromis diagramma*, *Haplochromis burtoni*, *Pundamilia nyererei*, *Maylandia zebra*, *Astatotilapia calliptera*, and *Mastacembelus armatus*) that happened gene fusion of *hsf5* and *rnf43* in vertebrates. Except for *M. armatus* which belongs to *Mastacembelidae*, the other seven species of bony fish all belong to *Cichlidae* and tropical fish. However, not all Cichlids *hsf5* and *rnf43* gene happened fusion, such as in *Archocentrus centrarchus* and *Neolamprologus brichard*. The phylogenetic tree analysis of the Hsf5 and Rnf43 amino acid sequences of other species and the Hsf5-rnf43 amino acid of eight species showed that Hsf5 and Rnf43 belong to two branches. The fusion protein Hsf5-rnf43 was not only clustered with Rnf43 but also clustered with Hsf5 (Figure 1).

The fusion way of heat shock transcription factor 5 and ring finger protein 43 genes

We selected three species to analyze how the genes *hsf5* and *rnf43* happened fusion. The gene structure was depicted to compare the differences. The length of the gene gap between *hsf5* and *rnf43* was 2484 bp from the termination codon (TAA) of *hsf5* to the initiation codon (ATG) of *rnf43* (Figure 2A). We can find that the fourth exon of *hsf5* and the second exon of *rnf43* happened splicing and skipped the last exon of *hsf5* and the first exon of *rnf43* (Figure 2A). The fourth exon of *hsf5* was spliced with the second exon of *rnf43* that spanned 70756 bp leading to a new fusion gene *hsf5-rnf43* (Data S1). We identified the result using the PCR method by designing three pairs of specific primers according to the last exon of *hsf5* and the first exon of *rnf43*. The primers used in this study are shown in Table S1. PCR results showed that the *hsf5* gene was highly expressed, *rnf43* was barely expressed, and *hsf5-rnf43* was moderately expressed in the testis, which means that *hsf5* and *rnf43* not only happened fusion but also were expressed independently (Figure 2B).

Spatio-temporal expression of heat shock transcription factor 5-ring finger protein 43

RT-PCR confirmed that *hsf5-rnf43* was specifically expressed in the testis of adult Nile tilapia but not detected in the gill, muscle, spleen, head kidney, kidney, liver, stomach, heart, brain, blood, and ovary (Figure 3A). The transcriptome data were analyzed during Nile tilapia ovary and testis development including stages of 5 days after hatching (DAH), 30 DAH, 90 DAH, and 180 DAH. The results showed that *hsf5-rnf43* began to express in the testis of 5 DAH, and it was significantly increased at 30 DAH, and the expression was stable at 90 DAH to 180 DAH. However, the expression of *hsf5-rnf43* was at an extremely low level during ovary development (Figure 3B).

Subcellular localization of heat shock transcription factor 5-ring finger protein 43

In the mature testis, the center of the seminiferous lobules was filled with spermatozoa, which concentrated in the seminal lobules after breaking through the cyst wall. Cysts with germ cells at different stages of development were also displayed. Primary spermatocytes with positive immunohistochemical staining showed yellow (Figure 4A). In the mature ovary, The cytoplasm was filled with yolk spheres and granules. Oocytes were surrounded by a follicle cell layer. Immunohistochemical staining showed almost no positive reaction in the ovary (Figure 4B). In the liver, as a negative control, the hepatocytes were centered on the central vein and arranged radially to form the hepatic cord. Immunohistochemical staining showed almost no positive reaction in the liver (Figure 4C).

Sex differences in methylation level of heat shock transcription factor 5-ring finger protein 43promoter

The CpG dinucleotides were not evenly distributed in the promoter region. A CpG island containing seven CpG dinucleotides existed in the *hsf5-rnf43* proximal promoter at positions −25 to −305 (nucleotide position relative to the transcription initiation site denoted as +1). The

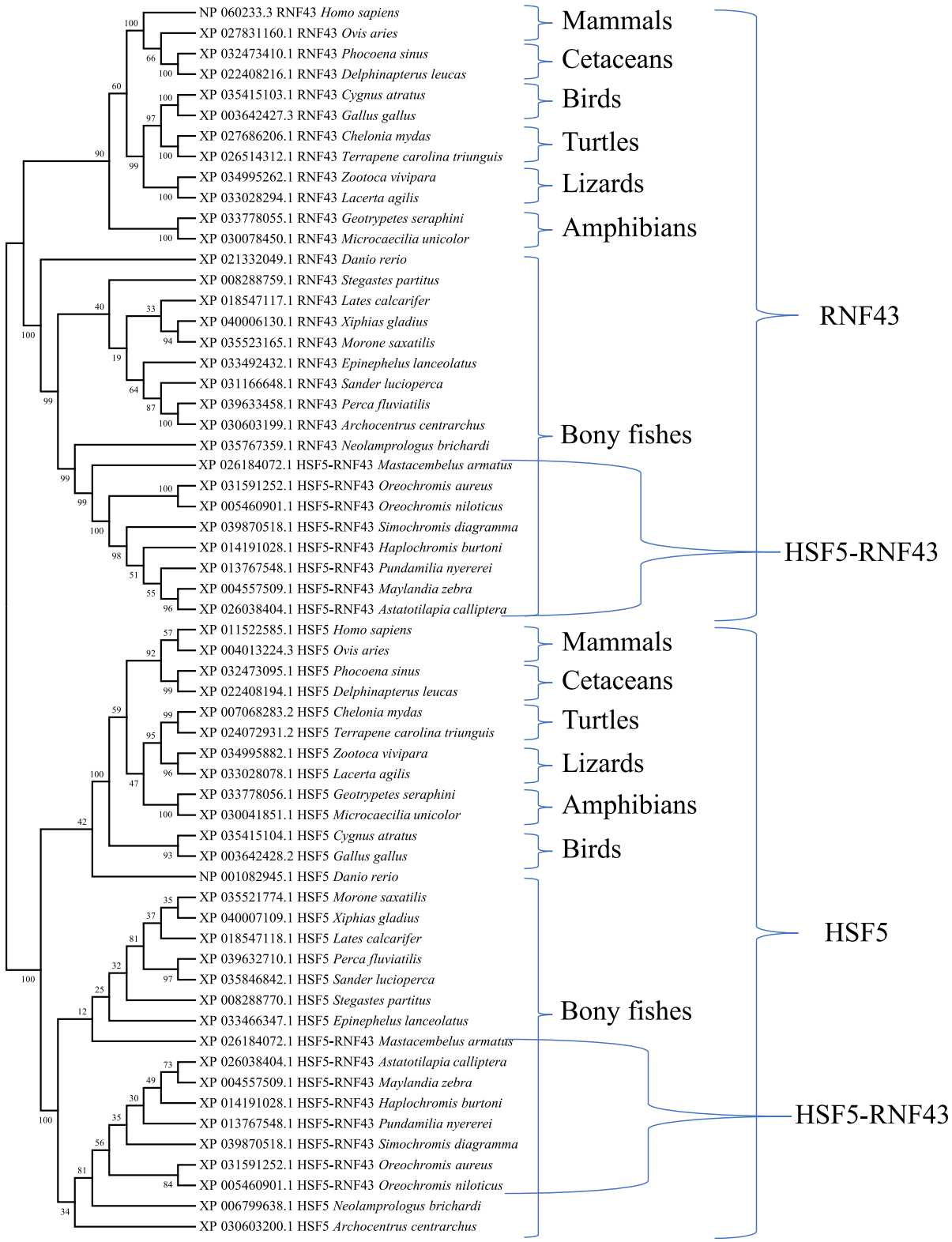

Figure 1. Phylogenetic analysis of Hsf5-rnf43

A phylogenetic tree based on the HSF5, RNF43, and HSF5-RNF43 amino acid sequence of multiple vertebrates was constructed using the Clustal W and the neighbor-joining method of MEGA 11. The node value indicates the percentage of 1000 replicates.

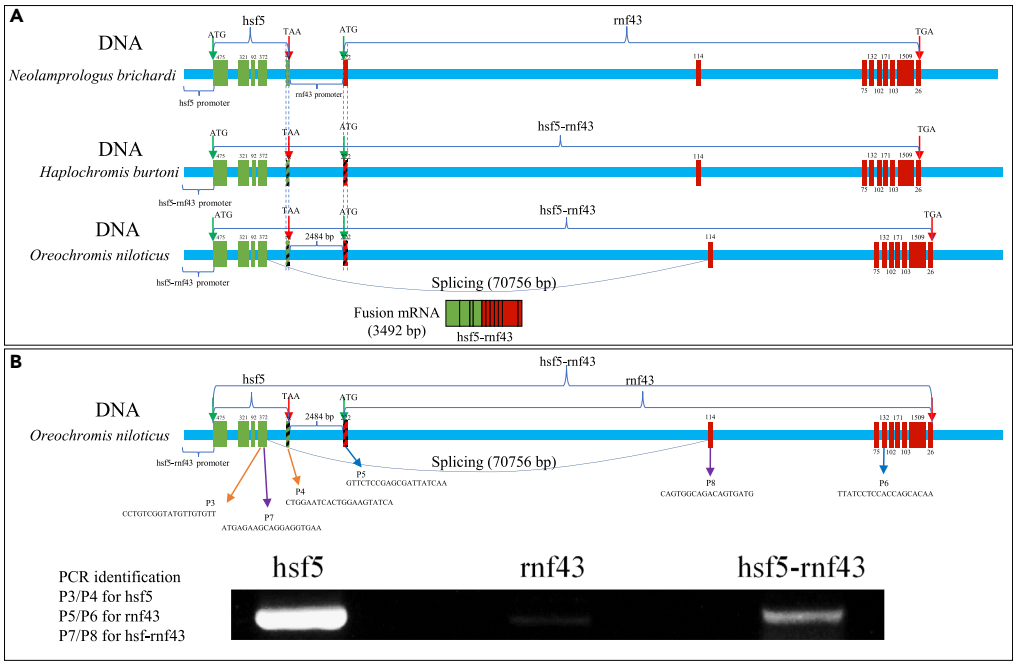

Figure 2. *Hsf-rnf43* gene identification

(A) Comparison of *hsf5*, *rnf43*, and *hsf5-rnf43* gene structure among three species. Green and red boxes represent exons of *hsf5* and *rnf43*, respectively. Blue long lines represent gene sequence, and introns are between boxes. Numbers on exons and other sites represent the length, and the unit is the base pair (bp).

(B) The fusion gene of *hsf5* and *rnf43* was identified by PCR. Boxes with black lines indicate skipped exons. P3/P4, P5/P6, and P7/P8 are paired primers to amplify the *hsf5*, *rnf43*, *hsf5-rnf43*, respectively.

predicted transcription factor binds to the sites and includes SRY-Box Transcription Factor 10 (SOX10), SOX3, Helicase Like Transcription Factor (HLTF), Homeobox A5 (HOXA5), Zinc Finger Protein 354C (ZNF354C), and Breast cancer type 1 susceptibility protein (BRCA1) (Figure 5A). Further study on the methylated level of CG sites was validated by BSP technology. The methylation percentage of *hsf5-rnf43* is lower in the testis (21.43 $\pm$ 7.65) than in the ovary (77.14 $\pm$ 2.34). The methylation levels of -25, -37, and -54 sites had the most significant difference. The BSP result trend was consistent with the data of MeDIP-Seq obtained from our previous study (Figure 5B). Reversely, the *hsf5-rnf43* was highly expressed in the testis but barely detected expressed in the ovary (Figures 5A and 5C). The activation of the *hsf5-rnf43* promoter was determined by Dual-luciferase reporter assay. Deleting the fragment of -25 to -305 sites significantly reduced the basal activity of *hsf5-rnf43* promoter (decrease to 47.67%). This result suggested that the region, which includes the above transcription binding sites, is essential for the regulation of *hsf5-rnf43* mRNA transcription. (Figure 5D).

DNA methylation and mRNA expression level of heat shock transcription factor 5-ring finger protein 43under high-temperature induced masculinization

The DNA methylation level of *hsf5-rnf43* promoter CpG island and the expression level of *hsf5-rnf43* mRNA under the conditions of high-temperature induced masculinization during the thermosensitive period from 5 DAH to 17 DAH were analyzed by DNA methylome and RNA transcriptome. We found that DNA methylation can regulate *hsf5-rnf43* mRNA expression in the early development of Nile tilapia. The DNA methylation level of *hsf5-rnf43* promoter CpG island was opposite to the expression level of *hsf5-rnf43* under high-temperature induced masculinization. The results showed that DNA methylation regulated *hsf5-rnf43* expression in the early development of Nile tilapia and was affected by high temperature during the thermosensitive period of Nile tilapia sex differentiation (Figure 6A). We also performed a correlation analysis between DNA methylation level and *hsf5-rnf43* expression level, which showed a strong negative correlation of 0.78 (Figure 6B).

DISCUSSION

Gene fusion is a process by which the complete or partial sequences of two or more distinct genes are fused into a single chimeric gene or transcript. The splicing and translation of such RNAs can lead to a new, fused protein, having domains from both original proteins.[9,10] The genes *hsf5* and *rnf43* happened fusion to form *hsf5-rnf43* gene in Nile tilapia according to our results. And the Hsf5-rnf43 protein kept the domains from both Hsf5 and Rnf43. In the region of *hsf5*, there was an HSF DNA-bind domain. Heat shock factors are a family of transcription factors involved in differentiation, development, reproduction, and stress-induced adaptation by regulating temperature-controlled *heat*

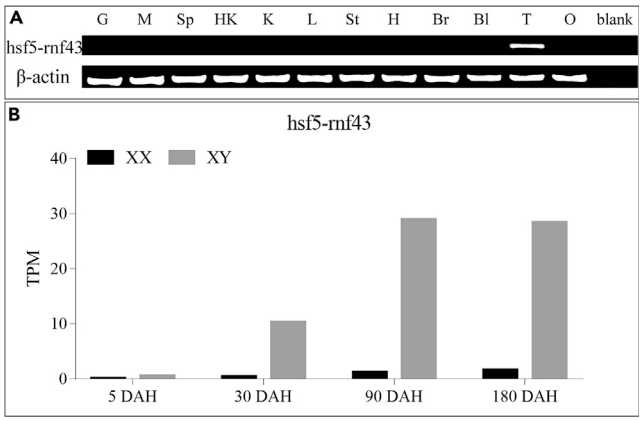

Figure 3. Spatio-temporal expression of *hsf5-rnf43*

(A) Tissue distribution of *hsf5-rnf43*. G: gill; M: muscle; Sp: spleen; HK: head kidney; K: kidney; L: liver; St: stomach; H: heart; Br: brain; Bl: blood; T: testis; O: ovary.
(B) Expression profile of *hsf5-rnf43* at different developmental stages. DAH: days after hatching; TPM: Transcripts per kilobase of exon model per million mapped reads. XX and XY represent female and male, respectively.

shock protein (hsp) genes and other non-*hsp* genes as well.[20] Therefore, the temperature may be an important environmental factor to regulate *hsf5-rnf43* gene expression. We found that the expression of *hsf5-rnf43* was increased under the conditions of high-temperature induced masculinization during the thermosensitive period from 5 DAH to 17 DAH. And *hsf5* is necessary for spermatogenesis and fertility in *D. rerio* and mice males.[11,12] In the region of Rnf43, ZNRF-3 ecto, and RING finger domains are conserved among vertebrates. ZNRF-3 is a transmembrane E3 ubiquitin ligase that antagonizes Wnt signaling, the signaling system used to mediate RSPO protein actions. This domain is known as the ectodomain.[21] A RING finger typically binds two zinc atoms with its Cys and/or His side chains in a unique "cross-brace" arrangement,

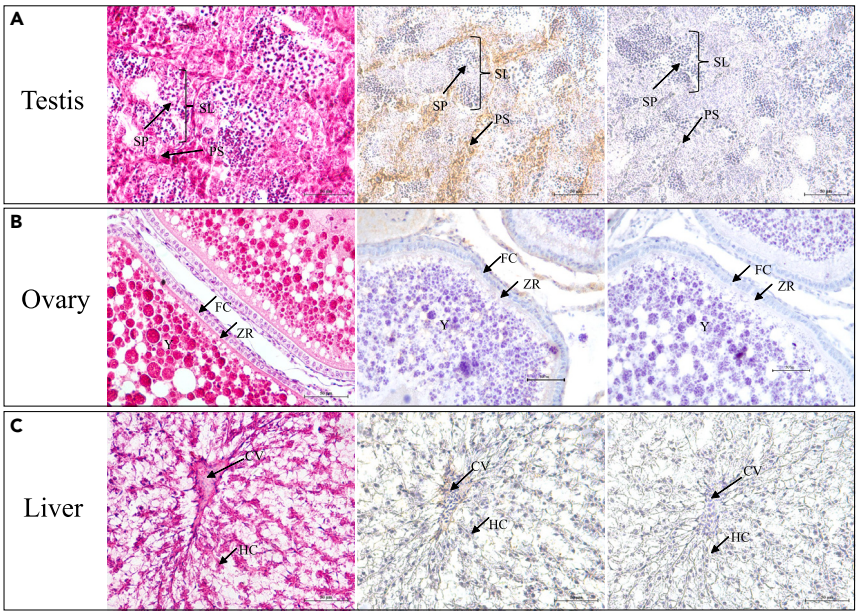

Figure 4. Subcellular localization of Hsf5-rnf43

(A) Hematoxylin and eosin staining in the testis (left figure). Immunohistochemistry staining with Rnf43 antibody in the testis (middle figure). Immunohistochemistry staining without Rnf43 antibody in the testis (right figure).
(B) Hematoxylin and eosin staining in the ovary (left figure). Immunohistochemistry staining with Rnf43 antibody in the ovary (middle figure). Immunohistochemistry staining without Rnf43 antibody in the ovary (right figure).
(C) Hematoxylin and eosin staining in the liver (left figure). Immunohistochemistry staining with Rnf43 antibody in the liver (middle figure). Immunohistochemistry staining without Rnf43 antibody in the liver (right figure). Abbreviation: PS, primary spermatocytes; SP, spermatid; SL, seminiferous lobule; FC, follicular cell; Y, yolk; ZR, zona radiata; CV, central vein; HC, hepatic cell. The original microscopic images were magnified 400×.

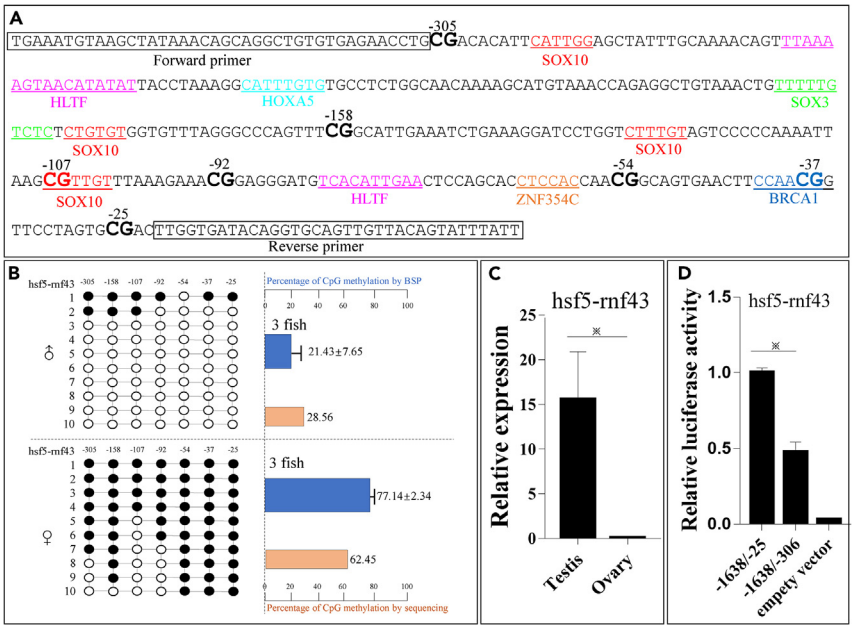

Figure 5. DNA methylation analysis of CpG islands in the promoter region of the *hsf5-rnf43* gene

(A) CpG island is located in the promoter region (−305/−25) of the *hsf5-rnf43* gene. The underline represents the predicted transcription factor binding sites.

(B) CpG island methylation level was analyzed. Black solids and circles represent methylation and unmethylation sites, respectively. Blue bar represents the percentage of CpG methylation via bisulfite sequencing PCR. Orange bar represents the percentage of CpG island methylation by MeDIP-Seq.

(C) qPCR analysis of *hsf5-rnf43* gene expression between testis and ovary.

(D) The activity of the *hsf5-rnf43* promoter and CpG island were determined by Dual-luciferase reporter assay. ※ indicates a statistically significant difference at $p < 0.05$.

which plays a key role in the ubiquitination pathway.[22] The existence of *hsf5* and *rnf43* domains implied that *hsf5-rnf43* may involve in spermatogenesis and immune function in the testis of Nile tilapia.

Transcription of a gene usually ends at a regulated termination point, preventing the RNA-polymerase from reading through the next gene. There are reports suggesting that chimeric transcripts, formed by the transcription of two consecutive genes into one RNA, can occur in humans.[10] *Hsf5* and *rnf43* are two consecutive genes, with a gene gap of only 2484 bp. However, we only detected eight species of bony fish with *hsf5-rnf43* gene fusion. Except for *Mastacembelus armatus*, which belongs to Mastacembelidae, the other seven species of bony fish all belong to Cichlidae and tropical fish. Gene fusions have contributed significantly to the evolution of species by providing a continuous source of new genes.[9] The Hsf5-rnf43 protein contains heat shock elements and is closely related to temperature changes.[20] Perhaps the emerged

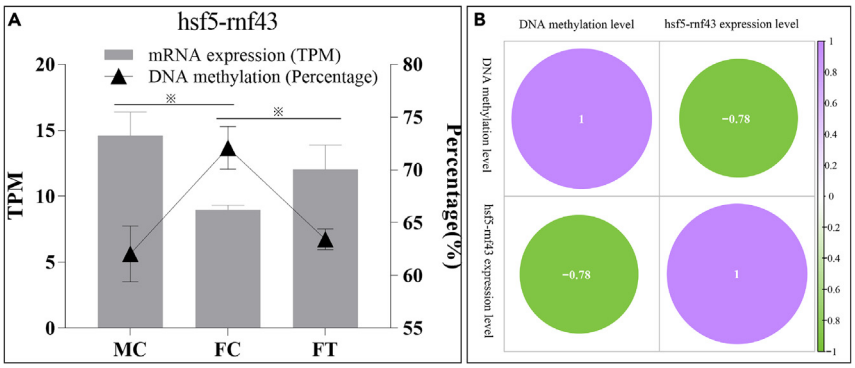

Figure 6. DNA methylation and mRNA expression level of *hsf5-rnf43* under high-temperature induced masculinization

(A) The left Y axis represents mRNA expression level of *hsf5-rnf43*. The right Y axis represents DNA methylation level of *hsf5-rnf43* promoter CpG island. MC: XY Male Control, 28°C. FC: XX Female Control, 28°C. FT: XX Females after high-temperature treatment during the thermosensitive period (36°C) from 5 to 17 days after hatching. TPM: Transcripts per kilobase of exon model per million mapped reads. ※ indicates a statistically significant difference at $p < 0.05$.

(B) The correlation analysis between DNA methylation level and *hsf5-rnf43* expression level.

fusion gene *hsf5-rnf43* is the result of fish evolutionary adaptation to the environment. The mechanism behind this phenomenon needs to be more experiments verified.

The transcription factor binding sites in the CpG island of the *hsf5-rnf43* promoter region, such as SOX10, SOX3, HLTF, HOXA5, ZNF354C, and BRCA1 were predicted. Deletion of this CpG island region can reduce promoter activity. HLTF,[23] HOXA5,[24] and BRCA1[25] have been reported as tumor suppressor genes. SOX10 significantly regulates the proliferation, migration, and apoptosis of tumors and is closely associated with the progression of cancer.[26] ZNF354C is reported as a transcriptional repressor that inhibits many genes and is related to esophageal squamous cell carcinoma.[27] These predicted five transcription factors suggested that *Hsf5-rnf43* may link to tumors and cancers. For the transcription factor SOX3, it has been reported that Sox3 is the *Oryzias dancena* male-determining factor, and loss-of-function mutations of Sox3 cause XY sex reversal.[28] Sox3 may also be a potential sex determination switch in the mammalian.[29] The cross-species comparison of the chromosomal location indicated that the *rnf43* gene was flanked by genes of *mtmr4*, *supt4h1*, and *tspoap1*. A chimeric transcript resulting from the fusion of RNF43 and SUPT4H1 was found to occur frequently in primary colorectal carcinoma. Knock-down of the expression of this RNF43-SUPT4H1 chimeric transcript was found to have a growth-inhibitory effect in colorectal cancer cells.[30] There was a *hsf5* gene in the upstream of *rnf43* gene in *D. rerio*, *X. tropicalis*, *L. agilis*, *C. mydas*, *G. gallus*, and *H. sapiens* except for *O. niloticus*. Hsf5 is necessary for spermatogenesis and fertility in *D. rerio* and mice males, and is mainly expressed in primary spermatocytes.[11,12] The analysis of gene collinearity and transcription factor binding sites has provided more reference information for the study of fusion gene *hsf5-rnf43*.

Nile tilapia as an important farmed fish, the growth rate of males is significantly higher than that of females.[1] All-male stocks are preferred as they improve the efficiency of commercial production. In this study, we found that the fusion gene *hsf5-rnf43* was sex-biased expressed in the testis and regulated by DNA methylation. DNA methylation often modifies the function of the genes and affects gene expression, which also affects sex determination and maintenance of fish. Global DNA methylation level increases in Nile tilapia gonads during high temperature-induced masculinization.[4] DNA methylation of the *cytochrome P450 family 19 subfamily A polypeptide 1a* (*cyp19a1a*) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass.[31] The DNA methylation inhibitor 5-aza-2-deoxycytidine reversed the natural sex change of *Monopterus albus*.[32] DNA methylation levels of the sex-related genes *dmrt1* and *cyp19a1a* are consistent with their expression quantities in *Paralichthys olivaceus*, and this epigenetic modification can influence the differential expression of genes in the gonads.[33] *Hsf5-rnf43* was identified as a sex-biased gene and regulated by DNA methylation such as *cyp19a1a*. We also found that DNA methylation regulated *hsf5-rnf43* expression in the early development of Nile tilapia and was affected by high temperature during the thermosensitive period of Nile tilapia sex differentiation from 5 DAH to 17 DAH. However, whether the *hsf5-rnf43* gene is related to sex determination and differentiation requires more experiments and research.

Hsf5-rnf43 was identified as expressed in the primary spermatocytes by IHC consistent with Hsf5 according to our results and ref. 11 and 12. Additionally, we analyzed the transcriptome data during Nile tilapia ovary and testis development including stages of 5 DAH, 30 DAH, 90 DAH, and 180 DAH. The 5 DAH is the critical period for sex determination and differentiation. The 30 DAH is the period of gonad morphological differentiation and the initiation of female meiosis. The 90 DAH is an important period for the initiation of male meiosis. The 180 DAH is an important period for male spermatogenesis and female yolk production.[34] We identified that *Hsf5-rnf43* began to express in the testis of 5 DAH and gradually increased during testis development. However, the expression of *hsf5-rnf43* was at an extremely low level during ovary development. The research can provide references for the molecular mechanism of sex determination and sex maintenance of Nile tilapia.

Limitations of the study

Although we have analyzed the expression and function of fusion gene *hsf5-rnf43* for the first time, we are aware that this study has some limitations. For example, 1) we identified that DNA methylation can regulate *hsf5-rnf3* sex-biased expression in the period of larva and adult Nile tilapia. However, it is unclear whether DNA methylation regulated the sex-biased expression of *hsf5-rnf43* throughout development from larva to adult in Nile tilapia. 2) We have shown that *hsf5-rnf43* is involved in sex determination and maintenance. However, the mutant *hsf5-rnf43* strain should be constructed to further identify how the gene *hsf5-rnf43* is involved in sex determination and sex maintenance.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108284.

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AUTHOR CONTRIBUTIONS

Yawei Shen and Hewei Jiang: conceptualization, methodology, data curation, formal analysis, writing – original draft, writing – review & editing, software. Tiantian Chen, Yufei Liu, Guokun Yang, and Xiaolin Meng: validation, methodology, visualization, and investigation. Adelino V. M. Canario: provided comments that improved the manuscript. Yawei Shen, Jinliang Zhao, and Xiaowu Chen: funding acquisition, conceptualization, supervision, project administration.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Rnf43 antibody	Sangon Biotech	Cat# D262139; RRID: AB_3073760
Anti-Rabbit IgG H&L (HRP)	Sangon Biotech	Cat# D110058; RRID: AB_2940954
Biological samples		
Oreochromis niloticus	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Tris-HCL	Invitrogen	Cat. No. 15568025
NaCl	Sigma-Aldrich	Cat. No. S5886
Sodium dodecyl sulfate	Sigma-Aldrich	Cat. No. L3771
EDTA	Invitrogen	Cat. No. 15575020
Proteinase K	QIAGEN	Cat. No. 19131
Xylene	Sigma-Aldrich	Cat. No. 534056
Bouin's solution	Sigma-Aldrich	Cat. No. HT10132
Ethanol absolute	Sangon Biotech	Order No. A500737
Neutral Balsam Mounting Medium	Sangon Biotech	Order No. E675007
Hematoxylin	Sangon Biotech	Order No. A600701
Eosin	Sangon Biotech	Order No. E607321
RNA*later*™	Invitrogen	Cat. No. 4427575
FuGENE® HD	Promega	Cat. No. E2311
Bgl II	Takara	Code No. 1021A
Hind III	Takara	Code No. 1060S
Critical commercial assays		
RNeasy Micro Kit	QIAGEN	Cat. No. 74004
PrimeScript™ RT Reagent Kit	TaKaRa	Code No. RR047A
EpiTect Bisulfite Kit	QIAGEN	Cat. No. 59104
iTaq Universal SYBR Green Supermix	Bio-Rad	Cat. No. 1725121
Dual-luciferase reporter assay Kit	Promega	Cat. No. E1910
Deposited data		
Raw data (transcriptome)	NCBI	BioProject: PRJNA170009
Raw data (methylome)	NCBI	BioProject: PRJNA769262
Raw data (transcriptome)	NCBI	BioProject: PRJNA564913
Gene sequence	NCBI	GenBank: OR591159
MeDIP–seq data	Chen et al.[8]	https://link.springer.com/article/10.1007/s10750-016-2823-6#Sec15
Experimental models: Cell lines		
HEK293T	ATCC	Cat. No. CRL-3216
Experimental models: Organisms/strains		
Oreochromis niloticus	This paper	N/A
Oligonucleotides		
See Table S1 for primer sequences	This paper	N/A
Recombinant DNA		
Plasmid: pGL3-basic	Promega	Cat. No. E1751

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Plasmid: pRL-TK	Promega	Cat. No. E2241
Software and algorithms		
Expasy	Duvaud et al.[35]	http://www.au.expasy.org/
BLAST tool	Tatusova et al.[36]	https://blast.ncbi.nlm.nih.gov/Blast.cgi
MEGA 11	Tamura et al.[37]	https://megasoftware.net/
Clustal W	Larkin et al.[38]	https://www.genome.jp/tools-bin/clustalw
Jalview	Waterhouse et al.[39]	https://www.jalview.org/
TMHMM Server	Moller et al.[40]	http://www.cbs.dtu.dk/services/TMHMM/
PredictProtein	Yachdav et al.[41]	https://www.predictprotein.org/
PROMO	Messeguer et al.[42]	http://alggen.lsi.upc.es/
JASPAR	Khan et al.[43]	http://jaspar.binf.ku.dk/
Transcription Element Search System	Schug et al.[44]	http://www.cbil.upenn.edu/tess
MethPrimer 2.0	Li et al.[45]	http://www.urogene.org/methprimer2/
Primer Premier	V6.0	http://www.premierbiosoft.com/primerdesign
GraphPad Prism	V7.0	https://www.graphpad.com/features

RESOURCE AVAILABILITY

Lead contact

Further information and requests of resources and reagents should be directed to and will be fulfilled by the lead contact, Xiaowu Chen (xwchen@shou.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) (BioProject: PRJNA170009, PRJNA769262, PRJNA564913) and are publicly available. Details of the gene sequence have been submitted to the NCBI GenBank database (GenBank: OR591159) and are publicly available.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICPANT DETAILS

The Nile tilapia in this study came from the Nile Tilapia Germplasm Resources Experimental Station of Shanghai Ocean University. We collected the adult male and female Nile tilapia at 180 DAH. They were separately raised in 2 water tanks (20 fish in each tank) at a temperature of 28°C, and fed formula feed twice a day. Five male fish and five female fish were randomly dissected and sampled. The ovary, testis, and liver were fixed in Bouin's solution (Sigma-Aldrich, St. Louis, MO, USA). Organs, including gill, muscle, spleen, head kidney, kidney, liver, stomach, heart, brain, blood, testis, and ovary were extracted and stored in RNA*later*™ Stabilization Solution (Invitrogen, Carlsbad, CA, USA) at -80°C. This present study of fusion gene *hsf5-rnf43* expression regulated by DNA methylation was affected by sex difference. Human embryonic kidney 293T (HEK293T) cells from ATCC (CRL-3216) were cultured in Dulbecco's Modified Eagle medium (GIBCO, Grand Island, NY, USA) with 10% fetal bovine serum (GIBCO) in 37°C incubator containing 5% CO2. Cells were tested for mycoplasma contamination and found to be contamination-free. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals. Protocols were approved by the Institutional Animal Care and Use Committee of Shanghai Ocean University and Henan Normal University.

METHOD DETAILS

Gene cloning and identification of hsf5-rnf43

DNA and RNA isolation and gene cloning were performed as described in the previous study.[8] Each sample macerated in 100 μl of lysis buffer (100 mM Tris–HCl, pH 8.0; 200 mM NaCl; 0.2% sodium dodecyl sulfate; 5 mM EDTA, pH 8.0; 100 mg/ml proteinase K) at 42°C overnight. The tissues were then heated for 10 min at 70°C to inactivate proteinase K. DNA extraction was performed by chloroform extraction and

iso-propanol precipitation. After being washed with 70% ethanol, the DNA was dissolved in 50 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Total RNA was isolated from each sample using RNeasy Micro Kit (QIAGEN, Duesseldorf, Germany). All RNA isolation procedures were performed in accordance with the manufacturer's protocol. DNA and RNA sample quality were checked by agarose gel electrophoresis, and quantification was accomplished by spectrophotometry. First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan). The full-length cDNA sequence of *hsf5-rnf43* was identified with primers (P1/P2) designed by Primer Premier 6.0 based on the *hsf5-rnf43* sequences from NCBI (Linkage Group LG10 - NC_031975.2 (6,666,006..6,777,000)). The gene name of sequence from NC_031975.2 (6,666,006..6,777,000) was called *rnf43* in GenBank, but we think it should be a fusion gene of *hsf5 and rnf43* called *hsf5-rnf43* according to our results. We designed three pairs of specific primers to identify the fusion gene *hsf5-rnf43*. The first pair of primers (P3/P4) were designed on the *hsf5* gene sequence that P3 was on the fourth exon and P4 was on the fifth exon to amplify *hsf5*. The second pair of primers (P5/P6) was designed on the *rnf43* gene sequence that P5 was on the first exon and P6 was on the fourth exon to amplify *rnf43*. The third pair of primers (P7/P8) was designed on the sequence spanning the *hsf5* and *rnf43* genes that P7 was on the fourth exon of *hsf5* and P8 was on the second exon of *rnf43* to amplify *hsf5-rnf43*.

Sequence analysis of hsf5-rnf43 gene

Hsf5-rnf43 protein sequence derivation and homologous species alignment analysis were performed using Expasy (http://www.au.expasy.org/)[35] and the BLAST tool in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi),[36] respectively. A phylogenetic tree of select Hsf5 and Rnf43 was constructed by using the neighbor-joining method in MEGA 11 with sequence alignment using Clustal W.[37] The multiple alignments of Hsf5 and Rnf43 amino acid sequences were built using Clustal W[38] and visualized through Jalview software.[39] The transmembrane (TM) region was predicted by TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/).[40] The putative conserved domain was detected using the online software PredictProtein (https://www.predictprotein.org/).[41] The promoter region of *hsf5-rnf43* was analyzed in terms of transcription factor binding sites by using PROMO (http://alggen.lsi.upc.es/)[42] and JASPAR (http://jaspar.binf.ku.dk/).[43] The putative transcription factor binding sites were predicted by the web-based software Transcription Element Search System (http://www.cbil.upenn.edu/tess).[44] MethPrimer 2.0 (http://www.urogene.org/methprimer2/) was used to predict the CpG island and design DNA methylation primers.[45]

Spatio-temporal expression of hsf5-rnf43

Reverse transcription PCR (RT-PCR) and real-time quantitative PCR (qPCR) were performed to analyze *hsf5-rnf43* gene expression using specific primers (P9/P10), *β-actin* was used as an internal reference gene with primers P11 and P12. All primers used in this study were shown in Table S1. qPCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using iTaq Universal SYBR Green Supermix (Bio-Rad) following our previous study.[46] All qPCR reactions were performed in a 20 μL reaction volume. The experimental parameters were as follows: initial denaturation of 20 s at 95°C, followed by 40 cycles of 5 s at 95°C, 15 s at 60°C, melting curve analysis at 65°C–95°C, and 0.5°C increment every 2–5 s. The relative expression of mRNA was evaluated by the $2^{-\triangle\triangle Ct}$ method with initial normalization of *hsf5-rnf43* against *β-actin*. We analyzed the transcriptome data during Nile tilapia ovary and testis development including stages of 5 DAH, 30 DAH, 90 DAH, and 180 DAH. The transcriptome data came from NCBI (BioProject: PRJNA170009) to identify the *hsf5-rnf43* gene expression along the development of Nile tilapia. For the samples information, 5 DAH (300 gonads pooled for each sex) was the critical time for sex determination and differentiation. 30 DAH (150 gonads pooled for each sex) was the initiation of germ cell meiosis and oogenesis in XX gonads. 90 DAH (3 gonads pooled for each sex) was initiation of germ cell meiosis or spermatogenesis in XY gonads. 180 DAH (3 gonads pooled for each sex) was the sperm maturation and vitellogenesis in XY and XX gonads.[34]

Subcellular localization

Three groups were prepared, including the testis, ovary, and liver groups, that were isolated from the development stage of 180 DAH. The testis was the experimental group compared to the ovary group. The liver was set as a negative control. Tissues were fixed with Bouin's solution (Sigma-Aldrich), dehydrated by ethanol (Sangon Biotech, Shanghai, China), transparent via xylene (Sigma-Aldrich), and embedded in paraffin. Tissue sections were sliced into 7 μm through rotary microtome for the subsequent experiment of hematoxylin and eosin (HE) staining and immunohistochemistry (IHC).

For HE, the tissue sections were deparaffinated with xylene for 10 min and rehydrated with ethanol series (100%, 95%, 85%, 75%, 50%) for 5 min. After rehydration, the tissue sections were washed with running water for 5 min and incubated with hematoxylin staining solution (Sangon Biotech) for 1 min. After being washed with running water for 5 min, the tissue sections were incubated with eosin staining solution (Sangon Biotech) for 20 s. Tissue sections were then washed with running water for 5 min, dehydrated with 50%, 75%, 85%, 95%, and 100% ethanol each for 3 min, and incubated with xylene for 10 min. Tissue sections were sealed with Neutral Balsam Mounting Medium (Sangon Biotech) and observed under the Nikon Ds-Ri2 camera (Nikon, Tokyo, Japan).

For IHC, tissue sections were firstly heated at 60°C for 60 min, deparaffinated, and rehydrated as described above. The tissue sections were performed antigen retrieval with sodium citrate buffer (pH 6.0) at 98°C for 20 min, eliminated endogenous unspecific binding with 3% H_2O_2 for 15 min, and blocked with 3% BSA-PBST for 30 min. The tissue sections were incubated with Rabbit Anti-RNF43 antibody (1:100, Sangon Biotech) and 3% BSA-PBST (with no Anti-Rnf43 antibody as a negative control) at 4°C overnight. After washing with PBST, tissue sections were incubated with Goat Anti-Rabbit IgG H&L (HRP) (1:200, Sangon Biotech) at 37°C for 30 min. Tissue sections were visualized with DAB reagent and counterstained with hematoxylin. Tissue sections were then dehydrated, transparentized, sealed, and observed as above.

We selected the Anti-Rnf43 antibody to further examine Hsf5-rnf43 localization in the testis, as it has been identified that Hsf5 is mainly expressed in primary spermatocytes.[11]

Cloning and DNA methylation analysis of hsf5-rnf43 promoter

The specific primers of *hsf5-rnf43* (P13/P14) (Table S1) were used to amplify their promoter sequence. Bisulfite sequencing PCR (BSP) was performed using the EpiTect Bisulfite Kit (QIAGEN) following the protocol using primer P15 and P16 for *hsf5-rnf43*. The total number of methylated cytosine was calculated as the average of the total number of methylated cytosine across the 10 sequenced clones. Three individuals were tested in each group. And we compared the BSP results to our previous study of whole-genome DNA methylation difference between the testis and ovary in Nile tilapia by using methylated DNA immunoprecipitation sequencing (MeDIP-Seq) technology.[8]

Plasmid construction, transfection, and Dual-luciferase assay

The fragment promoter sequence -1638/-25 of *hsf5-rnf43* was synthesized with primer (P17/P18) and cloned into pGL3-basic plasmid (Promega) using the double restriction enzyme digested by *Bgl* II and *Hind* III (Takara). The DNA sequence of the promoter with −1638/−306 region (deleted −305/−25 region) was synthesized with primer (P19/P20) cloned into the pGL3-basic plasmid (Promega) using the double restriction enzyme digested by *Bgl* II and *Hind* III (Takara). The activation of *hsf5-rnf43* promoter was determined by Dual-luciferase reporter assay. The constructed plasmids with pRL-TK (Promega) were co-transfected into HEK293T (CRL-3216) cells using FuGENE® HD (Promega) and seeded in 24-well plates for 24 hours. The luciferase activity measurement was performed as described in our previous work.[47] Firefly luciferase activity was normalized to Renilla luciferase activity. Three replicate experiments were set for each group.

DNA methylome and RNA transcriptome analysis

We analyzed the DNA methylome and RNA transcriptome during high-temperature-induced masculinization in sex-undifferentiated Nile tilapia gonad. The methylome and transcriptome data came from NCBI (BioProject: PRJNA769262, PRJNA564913) to identify the DNA methylation and mRNA expression level of *hsf5-rnf43* under high-temperature-induced masculinization. The data were classified into three groups, including FC (XX Female Control, 28°C), FT (XX Females after high-temperature treatment during the thermosensitive period (36°C) from 5 DAH to 17 DAH), and MC (XY Male Control, 28°C). The fish DNA and RNA samples from each group were extracted at 17 DAH. Each group was set with three experimental replicates and eighty fish for each replicate.[48]

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was calculated using GraphPad Prism 7.0. Values are expressed as mean ± SEM. Student's *t*-test was used to compare the difference in means between the two groups. $P < 0.05$ denotes statistical significance.