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Abbreviations[1]

[1] AA, amino acid; E, embryonic day; FAD, fadrozole; NAC, nascent-polypeptide-associated complex

ABSTRACT

In birds, the female is the heterogametic sex with one Z and one W-sex chromosome, while the male is the homogametic sex (ZZ). The chicken W chromosome is generally considered to be a degraded copy of the Z chromosome, comprising large repeat regions and, to date, 28 homologues of Z-chromosome protein coding genes. We have examined the expression profiles of all 28 Z and W-chromosome homologues in the developing gonads of the chick embryo. Here we report our analysis of one of these genes, BTF3 (basic transcription factor 3), which exhibits differential expression during gonadogenesis. Primers specific to *BTF3-W*, *BTF3-Z* and to a region common to both homologues, (BTF3-C), were designed to measure RNA expression by quantitative Polymerase Chain Reaction (q-PCR), and BTF3 protein expression was also analysed by Western-blotting and immunostaining. In addition, BTF3 RNA and protein levels were compared between the female gonad (ovary) and in female gonads following treatment to induce sex-reversal (testis). Our analysis showed that, overall, BTF3 RNA was expressed at higher levels in the female gonad than the male gonad, while BTF3-Z was subject to dosage compensation and expressed at similar levels in male and female embryos. However, BTF3 protein levels were actually higher in male gonads than in female gonads at embryonic day 6 (E6), suggesting regulation at the level of translation rather than transcription. Highest levels of BTF3 protein was seen in the medulla of the developing ovary and the sex cords of the developing testis, and BTF3 protein was principally localized to the cytoplasm of the somatic cells and germ cells of both male and female gonads. In addition, RNA levels, of both BTF3-Z and BTF3-W, in gonadal sex-reversed females, decreased to levels normally found in male gonads, linking BTF3 transcription with gonadal phenotype.

Key words: *BTF3*; chicken embryonic gonad; dosage compensation; W chromosome gene

INTRODUCTION

In birds, the sex chromosomes are designated as Z and W, and the males are the homogametic sex (ZZ) and the females the heterogametic sex (ZW). The chicken genome comprises 39 pairs of chromosomes: these are characterized as 6 pairs of macrochromosomes, 1 pair of sex chromosomes and 32 pairs described as either intermediate or microchromosomes. The Z chromosome is classified as a macrochromosome and carries around 1000 genes, while the W chromosome is a microchromosome and is generally regarded as a highly degraded copy of the Z chromosome [1,2], harbouring 76 loci of which 28 are protein coding [3]. For the majority of Z chromosome genes there is a dosage inequality between the sexes, with one copy in females and two copies in males [4]. In contrast, all W chromosome genes have homologous genes on the Z chromosome, and the protein-coding genes are highly homologous with their Z-copies (over 90% in most cases) [5,6]. These W genes are thought to be dosage sensitive factors that have important, but undefined, roles in development and that have been retained during evolution to match expression between females and males [1].

The chicken sex chromosomes are not homologous to the mammal sex chromosomes, and have evolved from a different pair of autosomes [6,7]. We have examined the expression profiles of all 28 W chromosome protein coding genes, and their Z chromosome homologues in the developing gonads of chick embryos, and identified a number that are differentially expressed in male and female gonads. Here we report our analysis of the expression profile of one of these genes, Basic Transcription Factor 3 (*BTF3*). Human *BTF3* has been designated as a general transcription factor that forms a stable complex with RNA polymerase II, and which is required for initiation of transcription. BTF3 has also be reported to bind to nascent polypeptide chains emerging from the ribosome and to block their interaction with the signal recognition particle

(SRP), so as to prevent the mis-translocation of non-secretory polypeptides to the endoplasmic reticulum. In addition, *BTF3* was reported to be related to the development of several types of cancer, via regulating transcription of tumor-associated genes and cell apoptosis [8-10].

As yet, the function of chicken *BTF3* remains to be determined. RNA-seq analysis (data not shown) showed that *BTF3-W* was highly expressed in both female germ cells and female embryonic gonads, and an on-line database ('Evo-devo mammalian organs', https://apps.kaessmannlab.org/evodevoapp/ [11]) shows that highest levels of expression of *BTF3-W* are found in the ovary, with lower expression in heart, kidney, liver and brain. The expression pattern of *BTF3-W* differs from that of human *BTF3*, which showed the similar levels of expression in all those tissues. This could suggest that chicken *BTF3* has acquired different or additional functions, perhaps involving gonadal development. Consequently, we established the RNA and protein expression profiles and the protein localization of chicken *BTF3* during gonadal development in the chick. In addition, we also investigated the effect of gonadal sex-reversal on *BTF3* RNA and protein expression.

Our analysis suggests that *BTF3* expression is associated with gonadal phenotype.

MATERIALS AND METHODS

Egg Incubation and Sample Collection

Freshly-laid fertile Hyline eggs were obtained from the National Avian Research Facility, U.K. They were incubated at 37.5°C under 60% humidity, blunt side up, and rotated every 30 minutes, and until the desired embryonic stage.

At embryonic day 6 (E6), E9, E12 or E18 , eggs were removed from the incubator and the embryos were then carefully dissected to expose the gonads. The gonads and tail tissue from E6

and E12 embryos were collected into tubes containing 10 µl of RNA-Bee (AMS Biotechnology) for RNA extraction. Gonads at E6, E9 and E12 were collected into 100 µl of RIPA buffer (Thermo Scientific, Cat No. 89900) for protein extraction. The gonads along with mesonephros at E6, E9, E12 and E18 were collected and fixed in 4% paraformaldehyde (PFA) in 12-well plate for 1 hour, for later cryosectioning and immunostaining. A small piece of tissue (wing or toe) was collected from each embryo to determine genetic sex [12].

Fadrozole Treatment of Chicken Embryos

E2.5 embryos were injected with Fadrozole (FAD), an aromatase inhibitor which causes gonadal sex reversal of female embryos [13]. A small hole was made in the blunt end of the egg and 0.1 mg FAD dissolved in PBS was injected into the air sac. The eggs were then sealed and reincubated until E12. Gonads samples were collected for RNA or protein extraction as described above.

RNA Isolation, cDNA Synthesis and Q-PCR

Total RNA was extracted from gonads and other tissues using RNA-Bee (AMS Biotechnology) according to the manufacturer's instructions. For E6 embryos, five pairs of gonads for each gender were pooled (5 pools for each sex). For E12 embryos, total RNA was extracted from single pairs of gonads (n=5 individuals for each sex). For E6 or E12 tails, total RNA was extracted from different individuals (n=5 for each sex). For FAD treatment, total RNA was extracted from single pair of gonads from different groups (control male, control female, FAD-treated male and FAD-treated female, n=5 for each group). First-strand cDNA was synthesized using a commercial Kit (GE Healthcare, Cat No. 27-9261-01) according to the

manufacturer's instructions. Sets of primers were designed to specifically amplify either the W chromosome *BTF3* (*BTF3-W)* or the Z chromosome *BTF3 (BTF3-Z),* or a region common to both *BTF3-W* and *BTF3-Z* (*BTF3-C)*. Primers were optimized for quantitative Real-Time PCR (qPCR) and the most efficient primer pairs selected (efficiency >95% <105%). Quantitative PCR analysis of the chicken hydroxymethylbilane synthase gene (*HMBS*) was used as the basis as an internal control. Primer sequences are listed in Supplementary Table 1. Relative RNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [14] and are presented as fold change over expression in E6 female gonads.

Protein Extraction and Western Blotting

Total protein was extracted from gonads into RIPA buffer according to the manufacturer's instructions (Thermo Scientific, Cat No. 89900). For E6 embryos, four pairs of gonads from the same gender were pooled (4 pools for each sex). For E9, two left or right side gonads from each gender were pooled (3 pools for each sex and for each side). For E12 embryos, protein was extracted from left or right side gonads from each individual (n=3 for each sex and for each side). For FAD treated embryos, protein was extracted from single pair of gonads from each treatment group (n=3 for each group). The nuclear and cytoplasmic protein fractions were prepared from E12 gonads (two pools for male and for female) using a 'NE-PER Nuclear and Cytoplamic Extraction Reagents' Kit (Thermo Scientific, Cat No. 78833) according to the manufacturer's instructions. Relative levels of BTF3 protein in individual samples were estimated using an 'Odyssey-based Western Blot Analysis' method as described (see protocol on http://biosupport.licor.com). Briefly, protein samples were subjected to electrophoresis on Bis-tris gels and then transferred to PVDF membranes. Following incubation with primary *BFT3*

antibody and fluorescently-labeled secondary antibodies, the membranes were imaged using an Odyssey imaging system. Scanned images were analyzed with 'Image Studio Lite Ver 5.2' software. *BTF3* antibody was from Novus (AF5747-SP) and *Tubulin* from Sigma (T3559) was used as loading control.

Cryosectioning and Immunostaining

Tissues were fixed in 4% paraformaldehyde/PBS for 1 h at 4 ℃, equilibrated in 15% sucrose/0.012M phosphate buffer overnight, embedded in 15% sucrose plus 7.5% gelatin/0.012M phosphate buffer (pH 7.2) and frozen at -70 ℃. Cryostat sections (10 µm) were prepared (OTF 5000 Bright Instruments), and collected onto glass slides (SuperFrost Plus, Thermo Fisher Scientific).

Immunohistochemistry was carried out as described previously [15]. Briefly, the slides were washed for 30 min in PBS at 37 °C and blocked in PBS containing 10% donkey serum, 1 % BSA and 0.3 % Triton X-100 for 2 h at 22–24 °C. Incubation with primary antibodies was carried out overnight at 4 °C, and the slides were washed in PBS containing 0.3 % Triton X-100, prior to incubation with secondary antibodies for 2 h at room temperature. Slides were then washed, in PBS containing 0.3 % Triton X-100 and the sections treated with Hoechst solution (10 µg ml^{-1}) for 5 min to stain nuclei.

Statistical Analysis

All values are expressed as mean ± standard deviation. SPSS 16.0 (SPSS China, Shanghai, China) was used to perform the Student's t-test or one-way analysis of variance for statistical significance of differences between or amongst different groups. $P < 0.05$ was considered

statistically significant.

RESULTS

Characterization of mRNA and Protein Sequences of Chicken BTF3

Sequences encoding *BTF3* are present on both the Z and the W sex chromosomes, and here are designated *BTF3-Z* and *BTF3-W* respectively. According to the latest version of the Ensembl database, chicken *BTF3-Z* (ENSGALG00000013512) produces a predicted transcript of 917 bp in length, encoding a protein of 162 AA (amino acid). While the *BTF3-W* transcript (ENSGALG00000000395.4, 1584 bp) is longer, the predicted protein is of a similar size (161 AA). A comparison of *BTF3-W* with *BTF3-Z* transcripts is depicted in Figure 1-A (detailed in Figure S1), and shows a sequence identity of 84.63%. The protein sequence alignment of *BTF3-W* and *BTF3-Z*, and human *BTF3* (two main isoforms, BTF3a and BTF3b) is shown in Figure 1-B. The predicted BTF3-Z protein is identical to that of human BTF3b protein, and differs from human BTF3a which contains an additional 44 AA N-terminal peptide. The amino acid sequences of BTF3-W and BTF3-Z differ only by 8 AA substitutions and a single AA deletion in the C-terminal region. Further analysis shows that all *BTF3* Open Reading Frames contain a conserved NAC (Nascent Polypeptide-Associated Complex) domain in the central portion (underlined in Figure 1-B).

The Spatiotemporal RNA Expression of Chicken BTF3 in Embryonic Tissues

Different sets of primers specific to *BTF3-W*, *BTF3-Z* or a region common to both transcripts (*BTF3-C*) were designed for qPCR quantification of expression profiles in both male and

female, gonad and tail tissues from E6 and E12 embryos. The positions of these primers on individual transcripts are shown in Figure 1-A and details are provided in Figure S2. Relative quantitation by qPCR is shown in Figure 2-A1. This shows that *BTF3-W* is only expressed in female tissues, with no amplification detected in any male tissue, as expected. The expression of *BTF3-W* was higher in female gonads than in tail tissue, and expression in female gonads increased significantly from E6 to E12 (P=0.013). We then investigated the expression of *BTF3-W* in an additional 7 female tissues at E12, all of showed a lower expression level than gonads (Figure S3-A). In contrast, expression of BTF3 Z was similar in males and females, and in gonads and in tails (Figure 2-A2 and Figure S3-B). The combined expression of *BTF3-W* and *BTF3-Z* (which was shown as *BTF3-C* in Figure 2-A3) was significantly higher in female gonads than in male gonads at E12, but similar in male and female gonads at E6.

The Protein Expression of Chicken BTF3 in Embryonic Gonads

Antibodies against the central portion of human BTF3 (which is 100% identical with chicken BTF3-Z) were used to detect the protein expression of chicken *BTF3* in this study (levels of chicken *Tubulin* protein were visualized to gauge loading variation). Following optimization of loading quantities (Figure S4), a series of Western Blot analyses were carried out on protein samples from male and female gonads collected at different embryonic stages (E6, E9 and E12, Figure 2-B and 2-C). As the predicted AA sequence of chicken BTF3-W is similar to BTF3-Z (Figure 1-B), WB analysis should represent the combined protein expression of *BTF3-Z* and *BTF3-W*. Surprisingly, given the RNA transcript levels, higher levels of *BTF3* protein were detected in male gonads than female gonads at E6 (Figure 2 B1). At E9 and E12 (Figure 2-B2 and B3), *BTF3* protein levels were similar between male and female left gonads. *BTF3* protein

levels were lower in female right gonads than female left gonads, and male right gonads. The expression level of *BTF3* protein decreased in both male and female left gonads with embryonic development (Figure 2-C1 and C2).

Localization of BTF3 Protein in Chicken Embryonic Gonads

We used immunostaining to establish the localization of *BTF3* protein in chicken embryonic gonads at different developmental stages. Staining with an anti-*BTF3* antibody revealed that the BTF3 protein could be detected in both male and female gonads at all embryonic stages tested (E6, 9, 12 and 18, Figure 3-A). Sections were co-stained with an antibody against P63 and with Hoechst (markers for germ cell and cell nuclei, respectively). Co-staining revealed that the *BTF3* protein was expressed throughout the gonad at early stages (E6 and E9), with higher levels in the medulla than in the surrounding epithelium. At later stages (E12 and E18), there are distinct morphological differences between the female gonad (ovary) and the male gonad (testis). In the ovary, *BTF3* protein was expressed uniformly across the medulla with lower levels in the cortex. In the testes, *BTF3* protein was mainly localized to (or around) the medullary sex cords. Confocal imaging confirmed that in both sexes, *BTF3* protein was expressed in both germ cells and somatic cells, and suggested that BTF3 was a cytoplasmic protein (Figure 3-B). Interestingly, co-staining with known male and female markers indicated that, in E18 testis, BTF3 was highly expressed in cells that express SOX9 (a marker for Sertoli cells). Western analysis of proteins in separated cytoplasmic and nuclear fractions (Figure 3-C) confirmed that chicken BTF3 protein was mainly restricted to the cytoplasm, with much lower levels found in the nucleus.

BTF3 Expression in Fadrazole-treated (sex reversed) Chicken Embryonic Gonads

As chicken *BTF3-W* RNA is expressed at higher levels in female gonads than in male gonads (Figure S3-A), embryos were subjected to Fadrazole-treatment (ovary to testis transformation) to determine whether *BTF3* expression is linked to gonadal phenotype. Figure S5 shows the morphology of E12 gonads in each treatment group (PBS-treated male and female gonads and Fadrazole-treated male and female gonads), demonstrating efficacy of treatment. Quantitative PCR analysis showed that the RNA expression of both *BTF3-W* and *BTF3-Z* in Fadrazole-treated female gonads was decreased compared with the control female (Figure 4-A and B), to the extent that the combined *BTF3* expression of Fadrazole-female gonads was similar to that seen in control male gonads (Figure 4-C). In addition, Fadrazole treatment also affected levels of *BTF3* protein, and resulted in similar levels of BTF3 in Fadrazole--treated female gonads and control male gonads. However, while Fadrazole-treatment resulted in a decrease in *BTF3* RNA, an increase in *BTF3* protein levels was observed. Overall, these analyses suggested that the expression of *BTF3* (*transcript and protein*) is linked to gonadal phenotype.

DISCUSSION

We have carried out an anlaysis of the expression of a sex-chromosome gene, *BTF3*, during gonadal development in the chick embryo. We show that both the *BTF3* RNA and protein are differently expressed between male and female gonads, and that these expression patterns are linked to gonadal phenotype. Our analysis also suggests that the role of *BTF3* is mainly confined to the cytoplasm.

Human *BTF3*, located on chromosome 5, has two protein isoforms (BTF3a and BTF3b) with molecular masses of 27 kDa and 22 kDa, respectively [16]. Human *BTF3* is one of the four

general transcription factors (*BTF1*, *BTF2*, *BTF3* and *STF*) which are reportedly required for initiation of transcription [17]. Previous studies have shown that while both BTF3a and BTF3b could bind to RNA polymerase II, only BTF3a was transcriptional active [16]. Human *BTF3* protein was also reported to be part of the nascent-polypeptide-associated complex (NAC) which prevents short ribosome-associated nascent polypeptides from inappropriate interactions with proteins in the cytosol [18], and was designated NACB. NACB was reported to be a cytoplasmic protein [17] and the molecular mass of NACB (22 kDa) [19], corresponds to that of BTF3b.

Little is known about chicken *BTF3*, other than the chromosomal location on the Z and W sex chromosomes. As shown in Figure 1B, while BTF3-Z is identical to human BTF3b, the predicted amino acid (AA) sequence differs from that of BTF3-W by eight substitutions and one deletion. Considering the highly conserved protein sequence with human BTF3b and the identical NAC domain (Figure 1-B), it's likely that chicken BTF3-Z also functions as a NAC protein，and this is supported by the fact that the majority of chicken *BTF3* protein is found in the cytoplasm (Figure 3-B and C). The sequence corresponding to the first 44 AA residues of human BTF3a is not present in the latest released version of the chicken genome. The fact that a small proportion of the *BTF3* protein is associated with the nucleus, means that it is theoretically possible that chicken *BTF3* may have a role in general transcription.

Although we first established that some Z chromosome genes were subject to a form of dosage compensation [20], it has been reported that most Z-linked genes are expressed at 1.4-1.8 fold higher in males (ZZ) than females (ZW) (due to the lack of global dosage compensation system akin to mammalian X inactivation) [4,21-24]. W-linked genes are generally considered to be dosage sensitive factors that have been retained to equalise male-female expression [1]. In this context, gonadal expression of chicken *BTF3* is unusual. While expression of *BTF3-W* is, of

course, restricted to females, *BTF3-Z* is expressed at similar levels in male and female gonads (Figure 2-A2). The combined expression of *BTF3-Z* and *BTF3-W* is higher (Figure 2-A1) in female than male gonads (Figure 3C). *BTF3-Z* transcription appears to be compensated so that it is expressed at similar levels in male and female gonads and in other tissues (Figure S3-B).

It is interesting to note that expression levels of both *BTF3-Z* and *BTF3-W* were reduced in gonads of female embryos treated with Fadrazole. Indeed the combined *BTF3* levels (Z+W) were reduced to levels usually found in male gonads. The reduction in BTF3 levels seen when embryonic ovaries are transformed into testes, suggests that *BTF3* levels are linked to gonadal differentiation. Development of the ovarian cortex in the chick embryo is estrogen-dependent and it is noteworthy that a recent study suggested that *BTF3* regulates *ERα* expression in luminal breast cancers, and showed that *BTF3* knockdown led to a substantially reduced expression of *ERα* and its transcriptional targets (*PGR*, *GREB1* and *IGFBP4*) [25].

However, there is a significant contradiction between the levels of chicken *BTF3* transcript and *BTF3* protein in the embryonic gonads. While total levels of *BTF3* RNA (*BTF3-C*) in male and female gonads was similar at E6, levels were significantly higher in the female at E12 (Figure 2-A3). In contrast, *BTF3* protein levels were significantly higher in the male gonad at E6 (Figure 2-B1) and similar in male and female gonads at E12 (Figure 2-B3). In addition, while *BTF3-W* RNA levels increased during gonadal development (Figure 2-A1), BTF3 protein levels in the female gonad decreased (Figure 3-C2). This contradiction is difficult to resolve as the antibody used in this study would bind to the predicted protein encoded by both *BTF3-Z* and *BTF3-W*. Of course it may be that the *BTF3-W* transcript is not translated and this possibility will require further study. However, even given that possibility, a degree of differential translation regulation would be required to explain the higher levels of BTF3 protein found in the male

gonad at E6, when BTF3 Z RNA levels are similar in males and females. In any event, it is of interest that the BTF3 protein is primarily located in the cytoplasm and that protein levels and patterns are clearly associated with gonadal morphology.

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FIGURE CAPTIONS

Figure 1. Characterization of RNA and protein sequences of chicken *BTF3*

A. mRNA structure comparison of chicken *BTF3-W* and *BTF3-Z*. The position of coding sequence (CDS), intron and Q-PCR primers specific to *BTF3-W*, *BTF3-Z* and their common region (*BTF3-C*) are indicated in the figure.

B. protein sequence alignment of human BTF3a, human BTF3b, chicken BTF3-Z and chicken BTF3-W. Mismatches are indicated with color background. The NAC (Nascent Polypeptide-Associated Complex) domain is underlined.

Figure 2. The spatiotemporal expression of chicken BTF3

A. Relative RNA expression of chicken *BTF3-W* (A1), *BTF3-Z* (A2) and *BTF3-C* (combined expression of *BTF3-W* and *BTF3-Z*, A3) by Q-PCR in chicken embryonic tissues.

B. Relative protein expression of chicken BTF3 by WB in gonads at E6 (B1), E9 (B2) and E12 (B3).

C. Relative protein expression of chicken BTF3 by WB in male (C1) and female (C2) gonads at different embryonic stages. F=female; M=male; E6 and E12 refer to embryonic day 6 and 12, respectively; L=left gonad; R=right gonad; columns with different letters on the top means significant difference ($P<0.05$); black spots refer to individual values in the corresponding group; the RNA and protein expression was calculated relatively to *HMBS* and *Tubulin*, respectively.

Figure 3. The localization of chicken *BTF3* in embryonic gonads

A. Expression of chicken BTF3 (red), P63 (green) and Hoechst (blue) in left gonadal sections by IHC at E6, E9, E12 and E18. F=female; M=male; scale bar=100 μm for all the panels.

B. Confocal images of expression of chicken BTF3 (red), P63 (green, top half), SOX9 (green, bottom half) and Hoechst (blue) in E18 male left gonads by IHC. Scale bars are annotated in the

figure separately.

C. Relative protein expression of chicken BTF3 by WB in nuclei and cytoplasm of E12 gonads. Nu=nuclei; Cyto=cytoplasm; M=male; F=female; the expression was calculated relatively to *Tubulin*.

Figure 4. *BTF3* expression in FAD-treated embryonic gonads

A/B/C. Relative RNA expression of chicken *BTF3-W* (A), *BTF3-Z* (B) and *BTF3-C* (combined expression of *BTF3-W* and *BTF3-Z*, C) by Q-PCR in control and FAD-treated embryonic gonads.

D. Relative protein expression of chicken BTF3 by WB in control and FAD-treated embryonic gonads. F=female; M=male; E12=embryonic day 12; WT=control; FAD=fadrozol; columns with different letters on the top means significant difference ($P<0.05$); black spots refer to individual values in the corresponding group; the RNA and protein expression was calculated relatively to *HMBS* and *tubulin*, respectively.

Figure 1

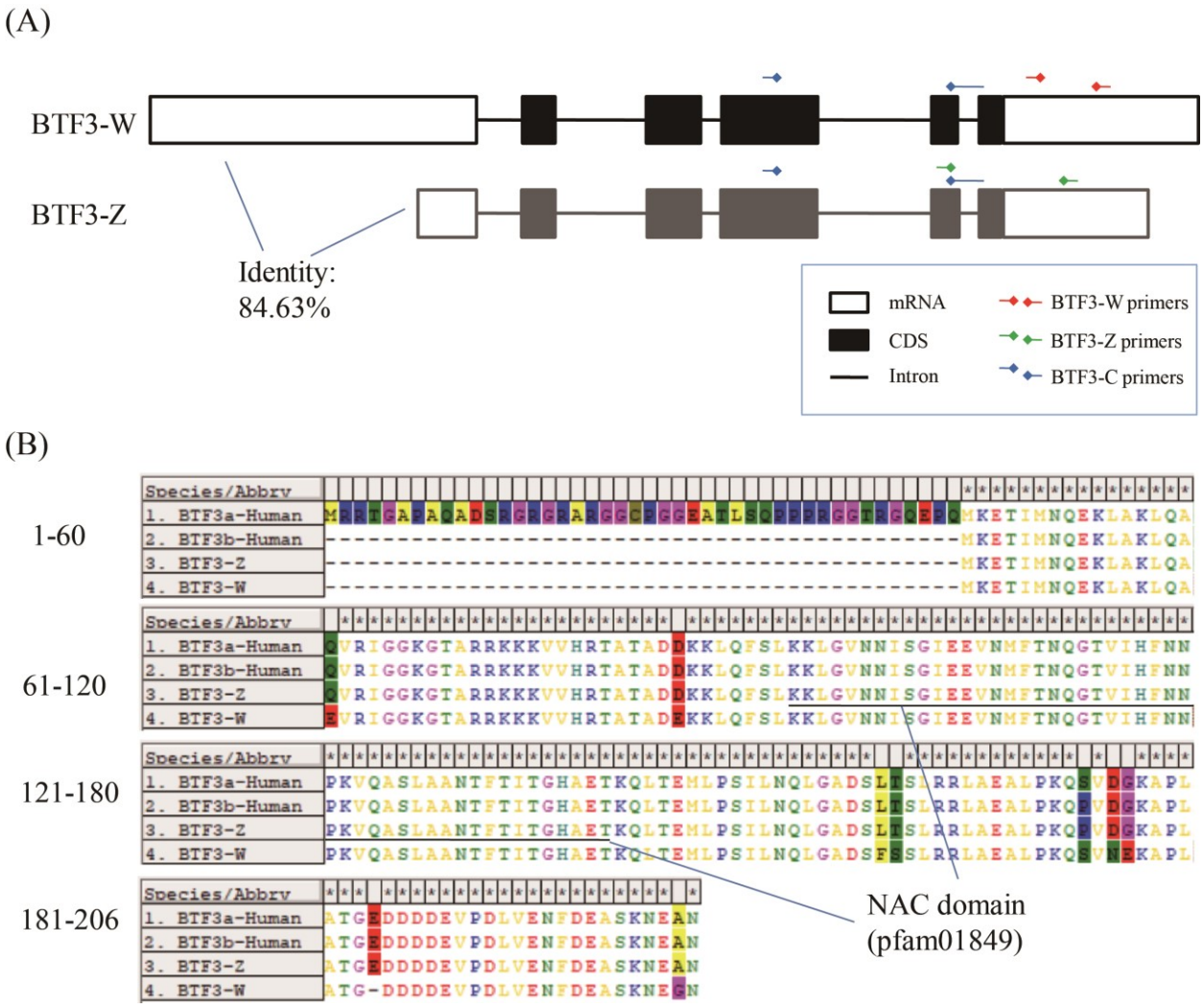

(A)
BTF3-W
BTF3-Z
Identity:
84.63%
mRNA
CDS
Intron
BTF3-W primers
BTF3-Z primers
BTF3-C primers
(B)
1-60
61-120
121-180
181-206
Species/Abbrv
1. BTF3a-Human
2. BTF3b-Human
3. BTF3-Z
4. BTF3-W
NAC domain
(pfam01849)

Figure 2

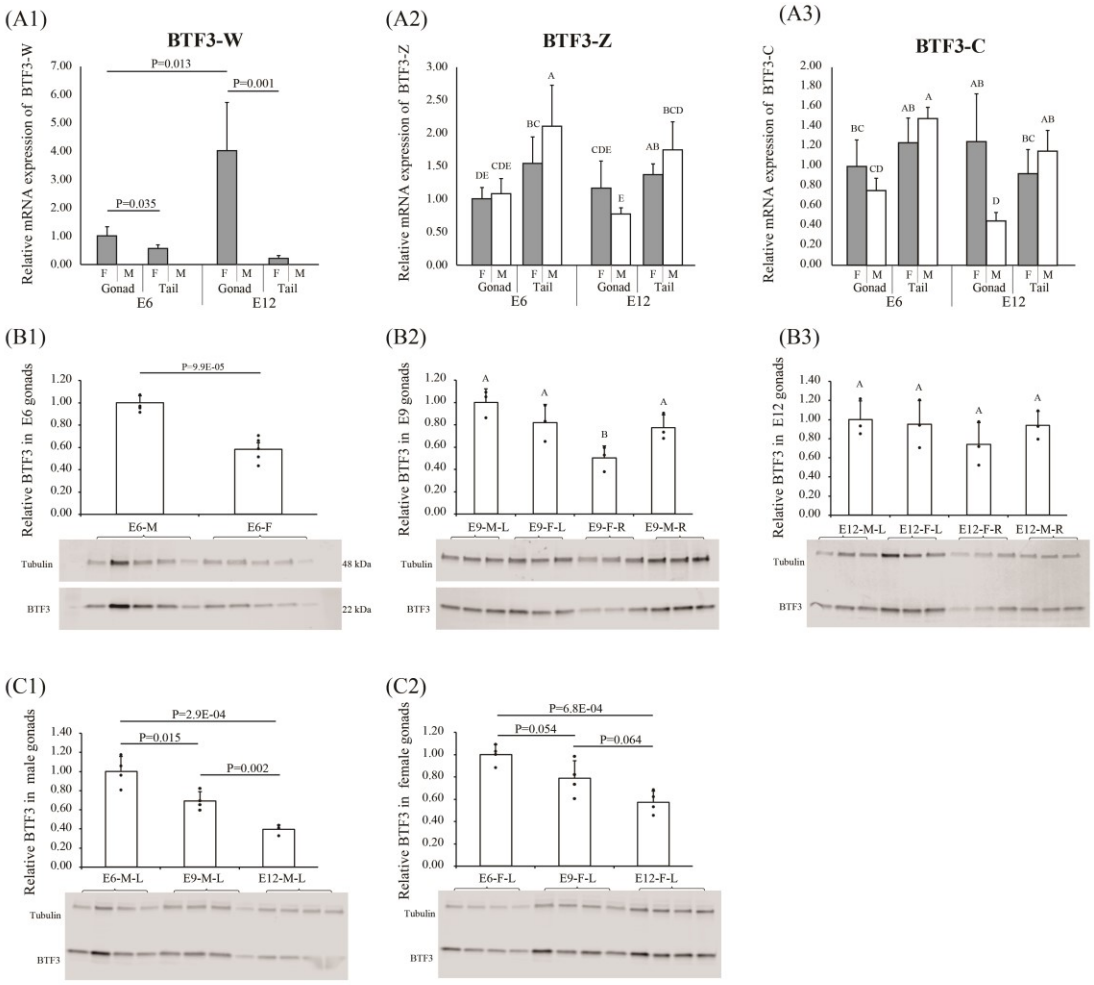

(A1)
BTF3-W
Relative mRNA expression of BTF3-W
P=0.013
P=0.001
P=0.035
F M F M F M F M
Gonad Tail Gonad Tail
E6 E12
(A2)
BTF3-Z
Relative mRNA expression of BTF3-Z
DE CDE BC A CDE E AB BCD
F M F M F M F M
Gonad Tail Gonad Tail
E6 E12
(A3)
BTF3-C
Relative mRNA expression of BTF3-C
BC CD AB A AB D BC AB
F M F M F M F M
Gonad Tail Gonad Tail
E6 E12
(B1)
Relative BTF3 in E6 gonads
P=9.9E-05
E6-M E6-F
Tubulin
BTF3
48 kDa
22 kDa
(B2)
Relative BTF3 in E9 gonads
A A B A
E9-M-L E9-F-L E9-F-R E9-M-R
Tubulin
BTF3
(B3)
Relative BTF3 in E12 gonads
A A A A
E12-M-L E12-F-L E12-F-R E12-M-R
Tubulin
BTF3
(C1)
Relative BTF3 in male gonads
P=2.9E-04
P=0.015
P=0.002
E6-M-L E9-M-L E12-M-L
Tubulin
BTF3
(C2)
Relative BTF3 in female gonads
P=6.8E-04
P=0.054
P=0.064
E6-F-L E9-F-L E12-F-L
Tubulin
BTF3

Figure 3

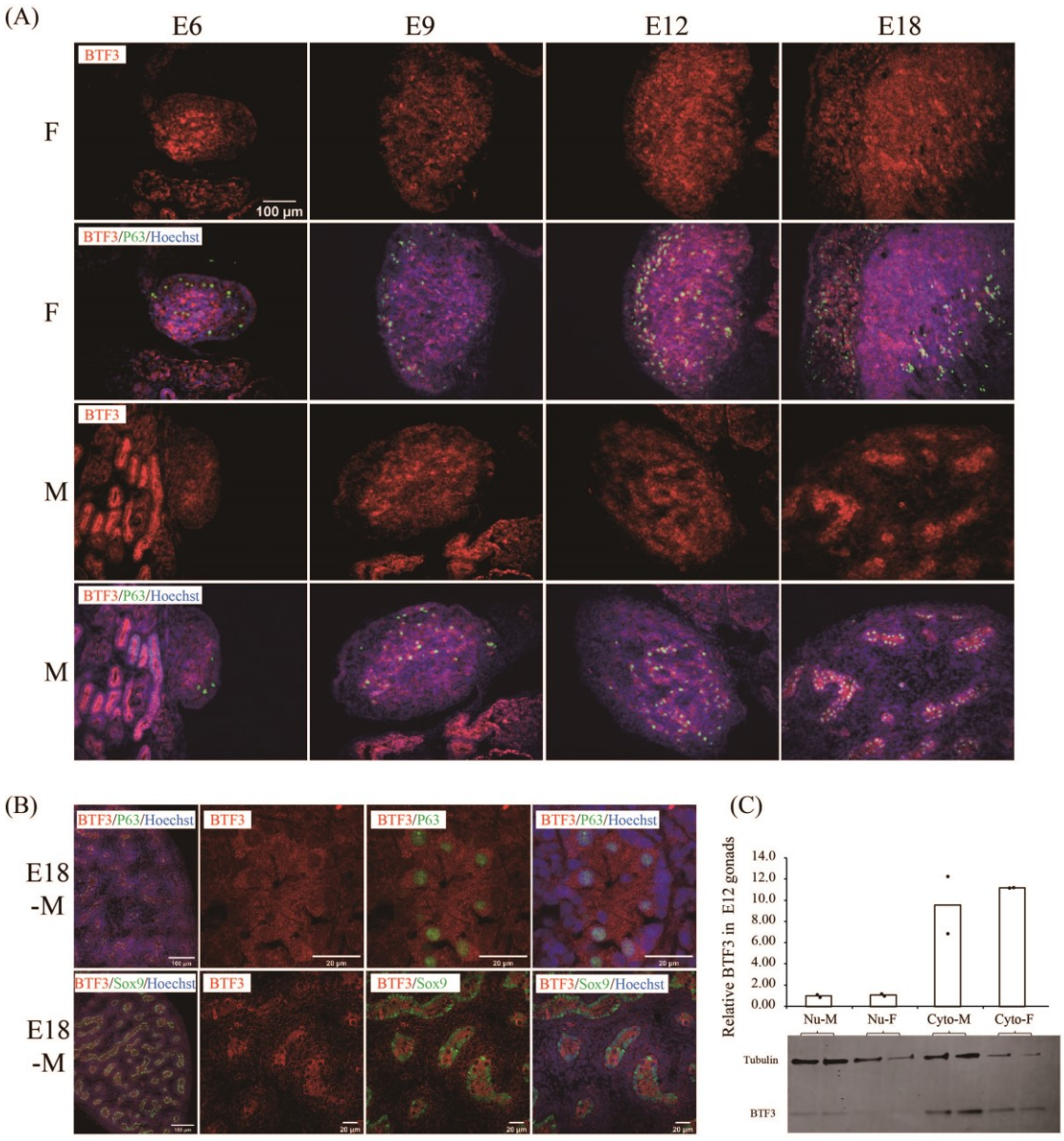
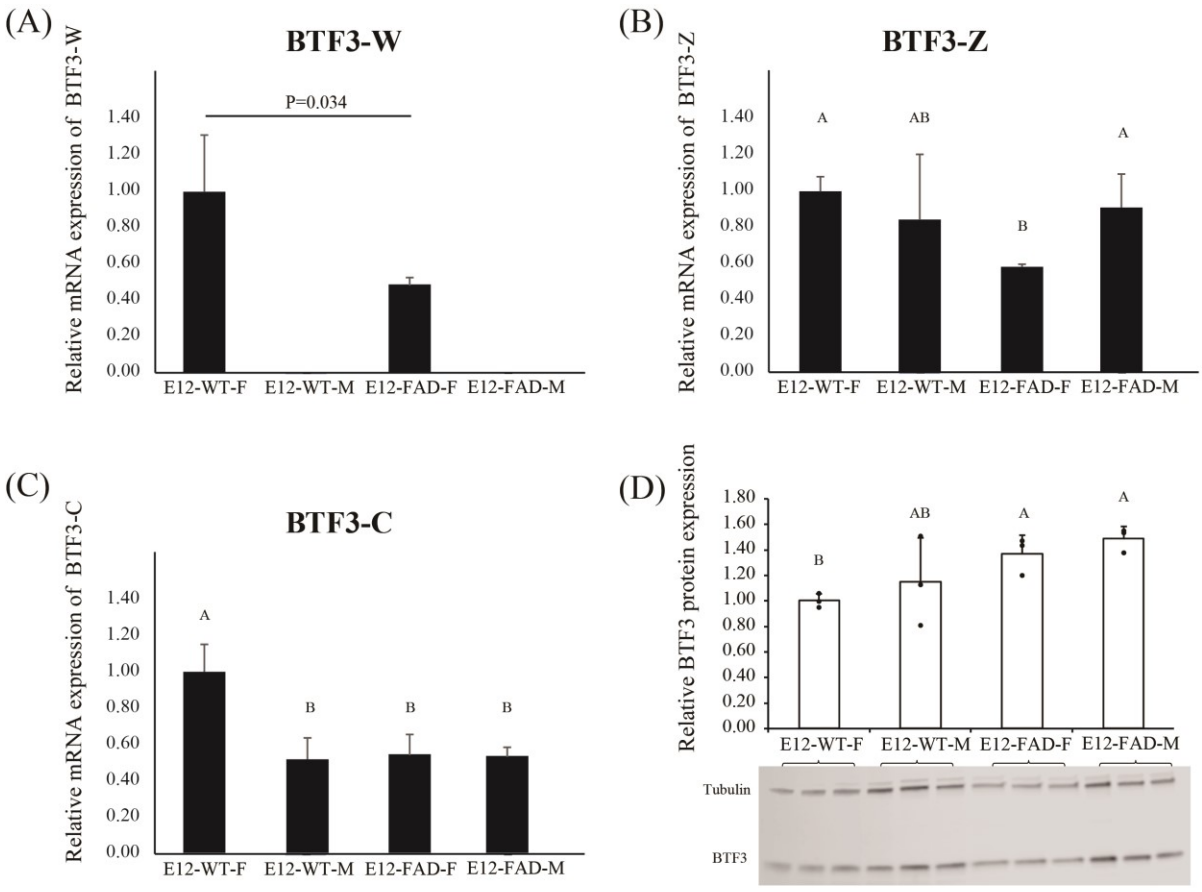

(A)
E6
E9
E12
E18
F
F
M
M
BTF3
BTF3/P63/Hoechst
100 μm
(B)
E18
-M
E18
-M
BTF3/P63/Hoechst
BTF3
BTF3/P63
BTF3/P63/Hoechst
BTF3/Sox9/Hoechst
BTF3
BTF3/Sox9
BTF3/Sox9/Hoechst
(C)
Relative BTF3 in E12 gonads
14.0
12.0
10.0
8.00
6.00
4.00
2.00
0.00
Nu-M
Nu-F
Cyto-M
Cyto-F
Tubulin
BTF3

Figure 4


(A)
BTF3-W
P=0.034
Relative mRNA expression of BTF3-W
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
E12-WT-F E12-WT-M E12-FAD-F E12-FAD-M
(B)
BTF3-Z
Relative mRNA expression of BTF3-Z
A AB B A
E12-WT-F E12-WT-M E12-FAD-F E12-FAD-M
(C)
BTF3-C
Relative mRNA expression of BTF3-C
A B B B
E12-WT-F E12-WT-M E12-FAD-F E12-FAD-M
(D)
Relative BTF3 protein expression
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
B AB A A
E12-WT-F E12-WT-M E12-FAD-F E12-FAD-M
Tubulin
BTF3

SUPPLEMENTARY MATERIALS

Supplementary Table 1. The sequences of primers for Q-PCR

Supplementary Figure 1. Detailed information of mRNA sequence comparison between *BTF3-W* and *BTF3-Z*

Supplementary Figure 2. Detailed information of Q-PCR primers for chicken *BTF3*

Supplementary Figure 3. Relative RNA expression of chicken *BTF3-W* and *BTF3-Z* in different embryonic tissues

Supplementary Figure 4. Optimization of antibodies for WB

Supplementary Figure 5. Morphology of E12 control gonads and fadrozol treated gonads