Sexually dimorphic expression of a chicken sex chromosome gene (VCP) reflects differences in gonadal development between males and females

Sexually dimorphic expression of a chicken sex chromosome gene (*VCP*) reflects differences in gonadal development between males and females

Xiao Lin[1,#], Yanfeng Fan[2,4,#], Debiao Zhao[3], Jason Ioannidis[3], Daoqing Gong[1], Long Liu [1,*], Michael Clinton[3,*]

*Correspondence: 597349037@qq.com; michael.clinton@roslin.ed.ac.uk

[1] College of Animal Science and Technology, Yangzhou University, Yangzhou , Jiangsu province, P.R. China

[2] Jiangsu Institute of Poultry Sciences, Yangzhou, P.R. China

[3] Division of Developmental Biology, The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin, UK

[4] Key Laboratory of poultry genetics and breeding in Jiangsu Province

Short Title: Expression profile of chicken VCP

[#] These authors contributed equally to this work.

Full name: Long Liu: Michael Clinton

Department: College of Animal Science and Technology, Yangzhou University, Yangzhou; The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh

Street Name & Number: Yangzhou, 225009, Jiangsu province, P.R. China; Roslin, Midlothian EH25 9PS, UK

Abstract

The chicken has a Z-W sex chromosome system, in which the males are the homogametic sex (ZZ) and the females the heterogametic sex (ZW). The smaller W chromosome is generally considered to be a highly degraded copy of the Z chromosome that retains around 28-30 homologous protein-coding genes' These Z-W homologues are thought to have important, but undefined, roles in development, and here we explore the role of one of these genes, *VCP* (Valosin Containing Protein) in gonadogenesis. We established RNA expression levels of both Z and W *VCP* homologues, the levels of VCP protein, and the cellular localization of *VCP* protein in male and female embryonic gonads during development. We also assessed the effects of female-to-male sex-reversal on *VCP* expression in developing gonads. The results showed that both *VCP* RNA and protein are expressed at higher levels in female than male gonads, and the expression levels of VCP protein and *VCP-Z* transcript, but not *VCP-W* transcript, are decreased in female-to-male sex reversed gonads. In addition, the spatial expression of VCP protein differs between male and female embryonic gonads: in testes, VCP protein is mainly confined to the medullary sex cords, while in ovaries, VCP protein is expressed throughout the medulla and at higher levels in the cortex. The results suggest that sexually dimorphic expression of chicken *VCP* reflects differences in gonadal morphology between sexes.

Keywords： Chicken, Embryonic gonad, *VCP*, W chromosome gene

1. Introduction

The sex chromosomes of birds are designated as Z and W, and the males are the homogametic sex (ZZ) and the females the heterogametic sex (ZW). The Z chromosome is classified as a macrochromosome and carries around 1000 genes, and due to the different copy numbers between male and female, most genes show a dosage inequality between the sexes. Sex determination in birds is thought to depend upon a dosage-based system, with the Z-linked *DMRT1* gene considered the best candidate for a chicken sex-determining gene [1]. However, the mechanism of chicken sex determination is still not fully understood, and it is possible that W-chromosome genes may also play a role. The W chromosome is generally regarded as a highly degraded copy of the Z chromosome, and harbours only about 28 protein coding genes [2, 3]. These Z-W homologues are thought to have important, but as yet, undefined roles in development [2]. We have examined the expression profiles of 28 W chromosome protein coding genes and their Z chromosome homologues in embryonic gonads, and identified a number of genes showing differential expression between males and females (data not shown). Here we report our analysis of the expression profiles of one of these genes, *Valosin Containing Protein (VCP)*. Mammalian studies have revealed that the autosomal *VCP* gene encodes a member of the AAA ATPase family of proteins, which plays an important role in numerous cellular processes including, protein degradation and turnover via the ubiquitin–proteasome system [4, 5].

To explore the possibility that the sex chromosome *VCP* played a role in chicken gonadogenesis, we established the expression profiles of both *VCP* homologues in developing male and female gonads. We also compared levels of VCP protein in male and female gonads and used immunohistochemistry to establish the cellular location of VCP protein in the developing ovary and testis. To determine whether differential expression was linked to gonadal development, we assessed the effects of sex reversal on *VCP* expression.

Our analysis suggests that changes in *VCP* expression reflect differences in morphology of male and female gonads.

2. Materials and methods

2.1 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, 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 a 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 [6].

2.2 Fadrozole Treatment of Chicken Embryos

E2.5 embryos were injected with Fadrozole (FAD), an aromatase inhibitor which causes gonadal sex reversal of female embryos [7]. 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.

2.3 RNA Isolation, cDNA Synthesis and Quantitative (real-time) Polymerase Chain Reaction (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 generated 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 sex-reversal study, 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 *VCP* (*VCP-W)* or the Z chromosome *VCP (VCP-Z),* or a region common to both *VCP-W* and *VCP-Z* (*VCP-C)* (Figure 1-A and details are provided in Figure S1). Primers were optimized for qPCR and the most efficient primer pairs selected (efficiency >95% <105%). Quantitative PCR analysis of the chicken hydroxymethylbilane synthase gene (*HMBS*) was used as internal loading control [8]. Primer sequences are listed in Supplementary Table 1. Relative RNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [9] and are presented as fold change over expression in E6 or E12 female gonads.

2.4 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 of each sex were pooled (5 pools generated for each sex). For E12 embryos, protein was extracted from left side gonads from each individual (n=5 for each sex). For FAD treated embryos, protein was extracted from single pairs of gonads from each treatment group (n=3 for each group). Nuclear and cytoplasmic protein fractions were prepared from E12 gonads (two pools for male and two pools 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 VCP protein in individual samples were estimated using an 'Odyssey-based Western Blot Analysis' method as described (see protocol on http://biosupport.licor.com). VCP antibody was from 4A Biotech Co.,Ltd. (ABIN2629813) and binding of Tubulin antibody (Sigma T3559) was used as loading control. The working concentration for VCP and Tubulin antibodies were both 1 μg/ml.

2.5 Cryosectioning and Immunostaining

Tissues were fixed in 4% paraformaldehyde/PBS for 1 h at 4 °C, 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 °C. 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 [10]. 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. The working concentration for the antibodies used were as follow: VCP -5 µg/ml, P63 (Abcam, ab124762) -2 µg/ml and Hoechst 33342 (Cambridge BioScience, ABCA2102792) - 1:100.

2.6 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 (Duncan's test) for statistical significance of differences between or amongst different groups. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Characterization of mRNA and Protein Sequences of Chicken *VCP*

Sequences encoding *VCP* are present on both the Z and the W sex chromosomes, and here are designated *VCP-Z* and *VCP-W* respectively. According to the latest version (GRCg7b) of the NCBI database, chicken *VCP-Z* (NM_001044664.1) generates a transcript of 2934 bp in length, encoding a protein of 806 AA (amino acid), while the *VCP-W* generates three potential isoforms (W1, XM_025144579.1, 2925 bp; W2, XM_025144580.1, 3190 bp; W3, XM_025144581.1, 3024 bp). A comparison of *VCP-W* with *VCP-Z* transcripts is illustrated in Fig. 1(A), and reveals a sequence identity of more than 90%. *VCP-W*1 and *VCP-Z* have similar gene structures (both have 17 exons and identical CDS lengths), while *VCP-W*2 and *VCP-*

*W*3 contain an extra exon (between the first and second exon of *VCP-W*1) of 153 bp and 135 bp, respectively. The additional exons alter the CDS and encode smaller proteins (761 AA for both *VCP-W*2 and *VCP-W*3).

The protein sequence alignment of VCP-W and VCP-Z, and human VCP (isoform 1, NP_009057.1) is illustrated in Fig. 1-B. The predicted VCP-Z and VCP-W1 proteins are the same length as human VCP protein, but contain 8 mismatches between VCP-Z and human VCP and 9 mismatches between VCP-W1 and human VCP. The amino acid sequences of VCP-W2 and VCP-W3 are identical with VCP-W1, except for the missing initial 43 AA. Further analysis shows that all VCP Open Reading Frames represent a conserved region (TIGR01243 for human VCP and cl27568 for chicken VCP -indicated in Fig. 1(B) encoding two ATPase domains connected by a short polypeptide linker.

3.2 The Spatiotemporal Expression of Chicken *VCP* transcript in Embryonic Tissues

Different sets of primers specific to *VCP-W* (common region of all 3 W transcripts), *VCP-Z* or a region common to both W and Z transcripts (*VCP-C*) were designed for qPCR quantification of expression in both male and female, gonad and tail tissues from E6 and E12 embryos. The results (Fig. 2-A1.) shows that, as expected, *VCP-W* is only expressed in female tissues with no amplification detected in any male tissue. Expression levels of *VCP-W* transcript were significantly higher in gonads than in tail tissue at E6, but decreased to levels equivalent to those in tail tissue at E12. By E12, *VCP-W* expression levels in gonads and tail were comparable to levels found in an additional eleven female tissues (highest expression level was found in cerebrum, Fig. S2-A). Expression levels of *VCP-Z* transcript were similar in male and female gonads at E6 and E12 (Fig. 2-A2). *VCP-Z* expression was higher in males than females except in E12 gonads, due to the dramatic decreased expression in E12 male gonads, compared with E6 male gonads (Fig. 2-A2). In contrast to *VCP-W*, *VCP-Z* expression levels varied considerably between different tissues, and in a number of tissues, *VCP-Z* was expressed at higher levels in males than in females (Fig. S2-B). The combined expression of *VCP-W* and *VCP-Z* (shown as *VCP-C* in Fig. 2-A3) was significantly higher in female gonads than in male gonads at both E6 and E12.

3.3 Expression of Chicken VCP Protein in Embryonic Gonads

An antibody against the C-terminal region of human VCP (which is 100% identical to chicken *VCP-Z* and *VCP-W*) was used to estimate levels of chicken VCP protein (levels of chicken Tubulin protein were visualized to gauge loading variation). Western Blot analyses were carried out on protein samples from male and female gonads collected at different embryonic stages (E6 and E12) (Fig. 2-B1 and 2-B2). As the predicted AA sequence of chicken VCP-W is similar to VCP-Z and the antibody binding region is identical (Fig. 1-B), our WB analysis should represent the combined protein expression of VCP-Z and VCP-W. In keeping with the higher combined RNA transcript levels (*VCP-C*) seen in female gonads, higher levels of VCP protein were also detected in female gonads than male gonads, at both timepoints ($P<0.01$, Fig. 2-B2).

3.4 Localization of VCP Protein in Chicken Embryonic Gonads

Immunostaining was used to establish the cellular localization of VCP protein in male and female embryonic gonads at different developmental stages (E6, E9, E12 and E18). Antibody staining revealed that the VCP protein could be detected in both male and female gonads at all embryonic stages tested (Fig. 3-A). Sections were also co-stained with an antibody against P63 and with Hoechst stain (markers for germ cell and cell nuclei, respectively). Immunostaining revealed that while the VCP protein was expressed in the medulla of both female and male gonads at early stage (E6), VCP was also found in the epithelial layer surrounding the female gonad. At later stages (E9, 12 and E18), there are distinct morphological differences between the female gonad (ovary) and the male gonad (testis). In the ovary, the epithelium thickens into a distinct cortical layer containing germ cells, while in the testis, sex cords coalesce around the germ cells in the medullary region. In the ovary, VCP protein was expressed uniformly throughout the medulla at all stages, and increased with development in the cortex. In the testes, elevated levels of VCP protein was mainly confined to the medullary sex cords. VCP protein was clearly present in the cytoplasm of both germ cells and somatic cells in the ovary and testis (Fig. 3-B). Additional Western analysis of proteins from separated cytoplasmic and nuclear fractions from E12 gonads (Fig. 3-C) confirmed that chicken VCP

protein was restricted to the cytoplasm and absent from the nucleus.

3.5 *VCP* Expression in Fadrazole-treated (sex reversed) Chicken Embryonic Gonads

As chicken *VCP* was expressed at different levels between female gonads and male gonads (Fig. 2), we assessed the effects of sex reversal (ovary to testis transformation) to determine whether *VCP* expression was linked to gonadal phenotype. Fig. S3 shows the gross 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 expression of *VCP-Z* but not *VCP-W* was significantly decreased in Fadrazole-treated female gonads compared with the control female (Fig. 4-A and B), leading to an overall decrease in combined VCP RNA levels (*VCP-C*, Fig. 4-C) and in protein (Fig. 4-D) expression. However, *VCP* expression in the sex reversed gonads was not reduced to the level of control male gonads. Overall, these analyses suggested that the expression of *VCP-Z* (transcript and protein) is linked to gonadal phenotype.

4 Discussion

The chicken sex chromosomes are designated Z and W. These Z/W homologues are thought to play important, but as yet undefined, roles in development, and we have established that a small number of these genes are differentially expressed in the male and female embryonic gonads [8]. Here we explore the possibility that one of these genes, *VCP*, is involved in sex-determination and/or gonadogenesis in the chicken.

We have established the expression levels of *VCP-Z* and *VCP-W* transcripts in male and female chick embryonic gonads at different stages of development and compared gonadal expression levels with expression in other tissues. We also compared VCP protein levels and cellular distribution in the developing testis and ovary. To explore the link between *VCP* and gonadal phenotype, we assessed the effects of gonadal sex-reversal on *VCP* expression.

We show that overall expression of *VCP* transcripts is higher in female gonads than in male gonads at both E6 and E12 of development. In contrast, *VCP* is expressed at similar levels in male and female tail tissue at both developmental stages. Sexually dimorphic *VCP* transcription is also reflected in levels of VCP protein with higher levels in female than in male gonads. However, given that *VCP* is highly expressed in all tissues we analysed, it seems unlikely that the sexually dimorphic expression that we observed in the developing gonads, underlies the sex-determining mechanism in chickens. Our IHC analyses show that the VCP protein is expressed at higher levels in the germ cells and the precursor steroidogenic and supporting cells of the ovary cortex and the testis sex cords than in the associated interstitial tissue. A link between gonadal morphology and *VCP* level is supported by the decrease in VCP protein seen as a result of ovary to testis sex reversal. It may be that sexually dimorphic expression of *VCP* in the gonads reflects differences in numbers of these specialised cell types in the embryonic testis and ovary. It is interesting that higher levels of *VCP* are seen in the female gonad than in the male gonad at E6, prior to the appearance of obvious morphological differences. IHC analysis reveals that this difference is due to VCP expression in the epithelium surrounding the gonads: VCP protein is absent in the testis epithelium but is present at high levels in the ovary epithelium. VCP expression in the female epithelium may reflect the early stages of ovarian cortex differentiation.

VCP (p97 in mammals) is a member of the AAA-ATPase superfamily and is associated with various cellular functions including, cell fusion, proliferation, secretion and protein turnover and degradation via the ubiquitin-proteasome pathway [4,11]. While *VCP* has been extensively studied in mammalian systems, there are few reports specifically relating to chicken *VCP*. These include a proteomic study designed to identify interacting partners of the Large conductance calcium-activated potassium (BK) channel, a genome study to identify genes associated with adaptation to high altitude environments, and a comparison of gene expression in the developing Mullerian ducts of male and female chick embryos [12-14]. These studies identified VCP as a hub protein in the BK-interactome, identified a *VCP* isoform selected for adaptation to high altitude environments, and established that *VCP* was expressed in female, but not male, embryonic

Mullerian ducts. Given the similarities between chicken and human VCP proteins, and considering the wide variety of cellular functions associated with VCP, it is possible that *VCP* expression levels simply reflect metabolic activity. For example, in the chick embryo, the Mullerian ducts in the male regress early in development while the ducts in the female are retained (the right duct later regresses): the sexually dimorphic expression of *VCP* reported [12] would reflect differences in metabolic activity in male and female Mullerian ducts. This would also accord with highest levels of *VCP* being associated with the sex cords in the testis and the cortex in the ovary.

We have established that chicken VCP protein is restricted to the cytoplasm, however, human VCP is reported to be found in both the cytoplasm and the nucleus of cells. It is possible that, compared to lower vertebrates, mammalian VCP has acquired additional nuclear functions, such as DNA-repair [4].

It is noteworthy that while gonadal expression of *VCP-Z* was reduced as a result of the ovary to testis sex-reversal, expression of *VCP-W* was not affected. This suggests that Z-chromosome and W-chromosome homologues are under different regulatory controls.

Statements

Statement of Ethics

All animal protocols were approved by the animal welfare committee of the Yangzhou University [permission number: SYXK(Su) IACUC 2012–0029], and comply with the associated guidelines.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

MC and LL conceived and designed the study. LX, YF, JI and DZ performed the experiments. LL wrote the paper. DZ, JI and MC reviewed and edited the manuscript. All authors read and approved the manuscript.

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Figure Legends

Fig. 1 Schematic depicting RNA and protein sequences of chicken *VCP*. A. mRNA structure comparison of chicken *VCP-W* (isoform 1, 2, and 3) and *VCP-Z*. The position of coding sequence (CDS), intron and Q-PCR primers specific to *VCP-W*, *VCP-Z* and their common region (*VCP-C*) are indicated. B. protein sequence alignment of human VCP (isoform 1), chicken VCP-Z and chicken VCP-W. Mismatches are indicated by coloured background. The conserved domain and antibody binding region are also indicated.

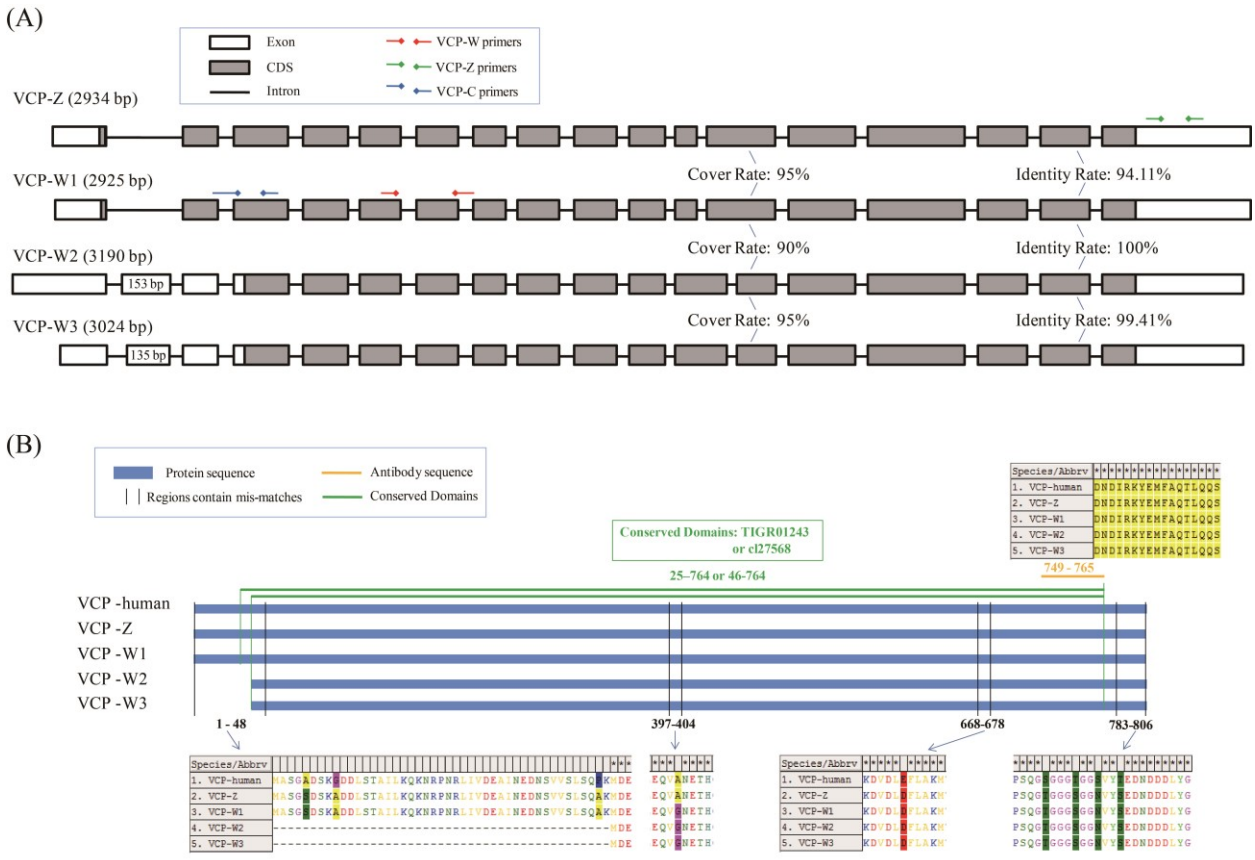
Fig. 2 Spatiotemporal expression of chicken *VCP*. A. Relative expression of *VCP-W* (A1), *VCP-Z* (A2) and *VCP-C* (combined expression of *VCP-W* and *VCP-Z*) (A3) RNA in chick embryo tissues by Q-PCR. B. Relative expression of chicken VCP protein in gonads at E6 (B1), and E9 (B2). F=female; M=male; E6 and E12 refer to embryonic day 6 and 12, respectively; columns with different letters indicate significant differences ($P < 0.05$); black spots refer to individual values in the corresponding group; VCP RNA and protein expression was calculated relative to levels of HMBS and Tubulin, respectively. Western images are shown below relevant graphs (the wrong symbol X means a false sample loading whose result was not included in the analysis).

Fig. 3 Localization of chicken VCP in embryonic gonads. A. Expression of chicken VCP (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; C=cortex; Me=medulla; SC=sex cord. B. A higher magnification of images of expression of chicken VCP (red), P63 (green, top panels) and Hoechst (blue) in E18 female and male left gonads by IHC. scale bar=200 μm for all the panels. C. Relative protein expression of chicken VCP in nuclei and cytoplasm of E12 gonads. Nu=nuclei; Cyto=cytoplasm; M=male; F=female; expression levels were calculated relative to Tubulin levels. Western image is shown below graph.

Fig .4 *VCP* expression in Fadrozol (FAD)-treated embryonic gonads. A/B/C. Relative expression of chicken *VCP* RNA: *VCP-W* (A), *VCP-Z* (B) and *VCP-C* (combined expression of *VCP-W* and *VCP-Z*)(C) in control (WT) and FAD-treated male (M) and female (F) embryonic gonads. D. Relative expression of chicken VCP

protein in control (WT) and FAD-treated embryonic gonads. F=female; M=male; E12=embryonic day 12; WT=control; FAD=fadrozol; columns with different letters indicate significant difference ($P<0.05$); black spots refer to individual values in the corresponding group; Western image is shown below graph; RNA and protein expression was calculated relative to HMBS and Tubulin, respectively.

Fig. 1


(A)
Exon
CDS
Intron
VCP-W primers
VCP-Z primers
VCP-C primers
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VCP-W1 (2925 bp)
VCP-W2 (3190 bp)
153 bp
VCP-W3 (3024 bp)
135 bp
Cover Rate: 95%
Identity Rate: 94.11%
Cover Rate: 90%
Identity Rate: 100%
Cover Rate: 95%
Identity Rate: 99.41%
(B)
Protein sequence
Regions contain mis-matches
Antibody sequence
Conserved Domains
Conserved Domains: TIGR01243
or cl27568
25–764 or 46-764
749 - 765
VCP -human
VCP -Z
VCP -W1
VCP -W2
VCP -W3
1 - 48
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668-678
783-806
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3. VCP-W1
4. VCP-W2
5. VCP-W3

Fig. 2

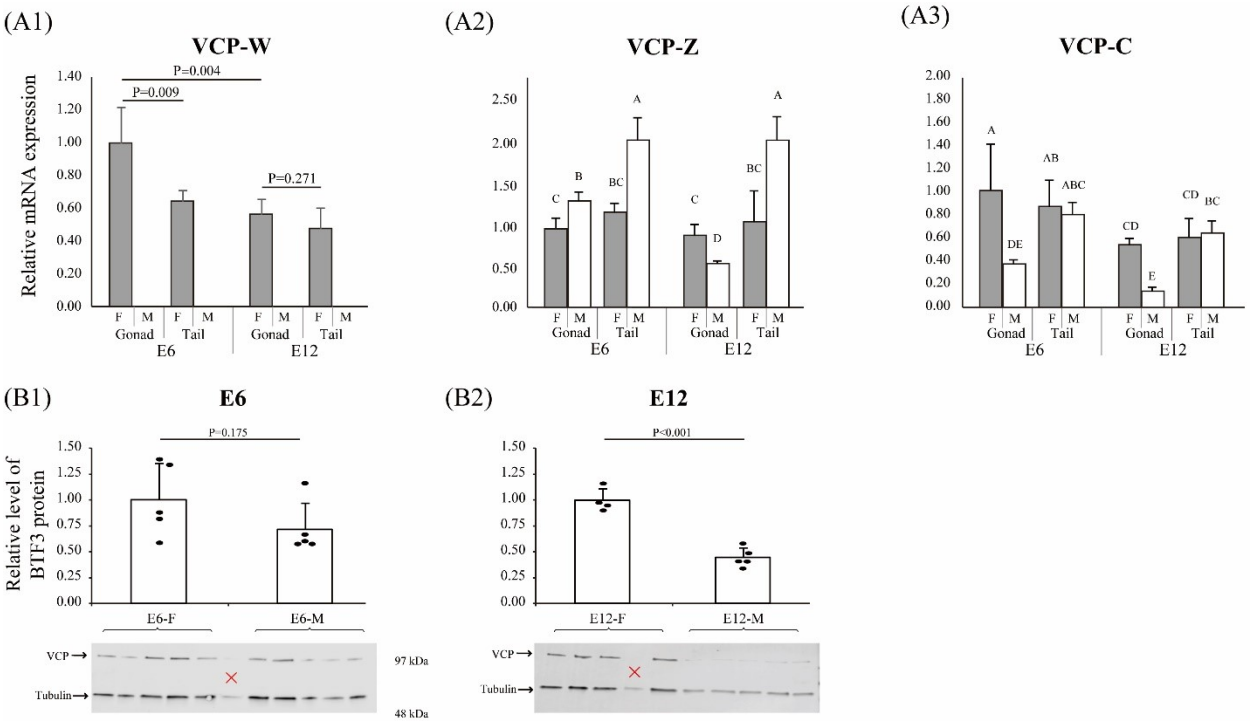

(A1)
VCP-W
Relative mRNA expression
P=0.004
P=0.009
P=0.271
F M F M F M F M
Gonad Tail Gonad Tail
E6 E12
(A2)
VCP-Z
C B BC A C D BC A
(A3)
VCP-C
A DE AB ABC CD E CD BC
(B1)
E6
Relative level of BTF3 protein
P=0.175
E6-F E6-M
VCP
Tubulin
97 kDa
48 kDa
(B2)
E12
P<0.001
E12-F E12-M

Fig. 3

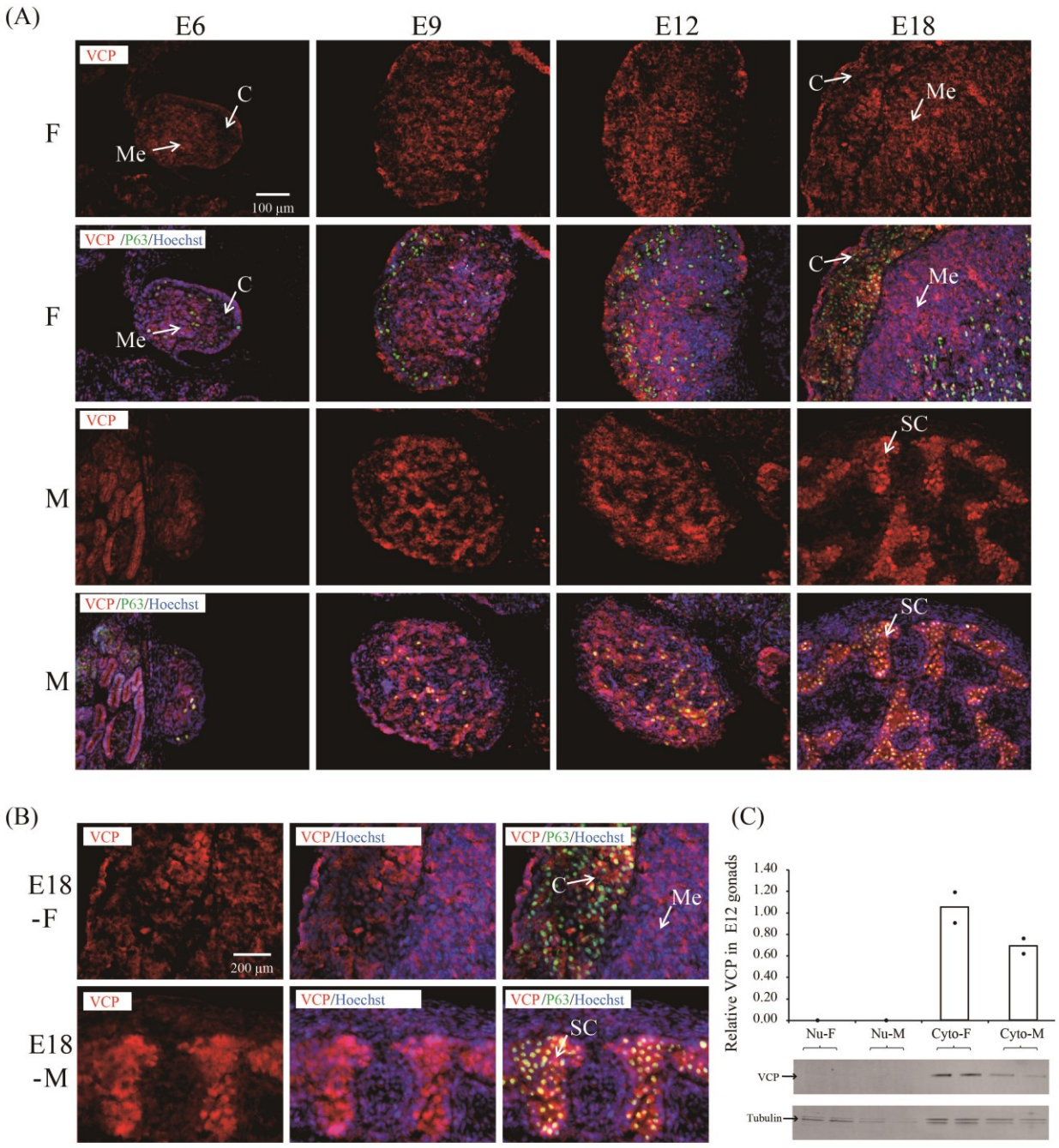
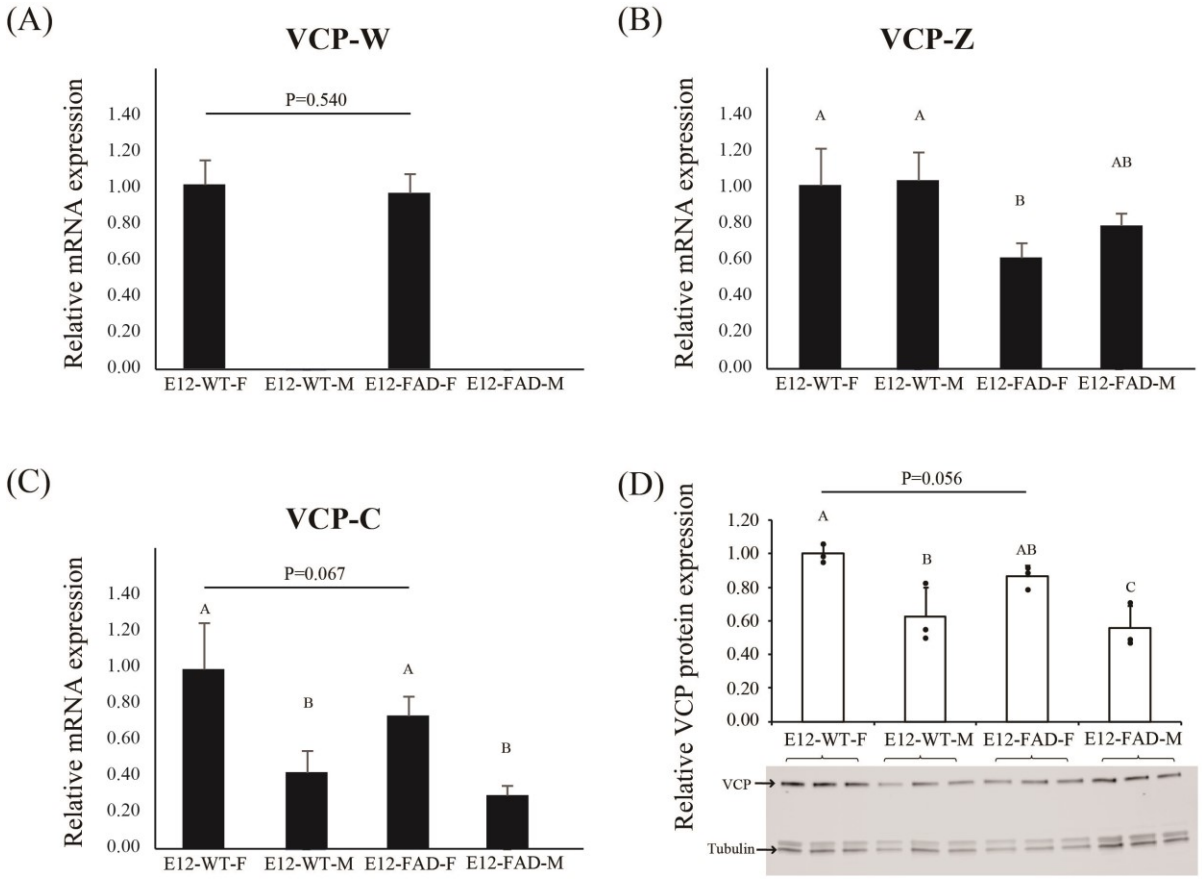

(A)
E6
E9
E12
E18
F
M
VCP
VCP /P63/Hoechst
C
Me
SC
100 μm
(B)
E18
-F
E18
-M
VCP/Hoechst
200 μm
(C)
Relative VCP in E12 gonads
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Nu-F
Nu-M
Cyto-F
Cyto-M
VCP
Tubulin

Fig. 4


(A)
VCP-W
P=0.540
Relative mRNA expression
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)
VCP-Z
A
A
B
AB
(C)
VCP-C
P=0.067
A
B
A
B
(D)
P=0.056
Relative VCP protein expression
VCP
Tubulin