Studies on the functional differentiation of *UBAP2* genes linked to the sex chromosomes in Japanese quail (*Coturnix japonica*)

ニホンウズラにおける性染色体連鎖遺伝子 *UBAP2* の機能分化に関する研究

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Contents

Abbreviations

BCA	bicinchoninic acid
CASI	Cell-autonomous sex identity
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GO	gene ontology
HH	Hamburger and Hamilton
ICC	Immunocytochemistry
IHC	Immunohistochemistry
NBT/BCIP	nitroblue Tetrazolium/5-Bromo-4-chloro-3-indolyl-phosphate
NCBI	National Center for Biotechnology Information
RIPA	radioimmunoprecipitation
RSEM	RNA sequencing by expectation maximization
RT-PCR	reverse transcription polymerase chain reaction
RNA-seq	RNA sequencing
STAR	spliced transcripts alignment to a reference
Tris	tris (hydroxymethyl) aminomethane
UTR	untranslated region
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PFA	paraformaldehyde
PGC	primordial germ cell
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
TBS	tris-buffered saline
TPM	transcripts per million
WISH	whole-mount *in situ* hybridization

General Introduction

In many species with separate sexes, two major sex chromosome systems have evolved. Mammals have an XX/XY system, in which males are heterogametic with the X and Y chromosomes, whereas birds have a ZZ/ZW system, in which females are heterogametic with the Z and W chromosomes. Although the sex chromosomes of mammals and birds are not orthologous, both evolved from ancestral autosomes[1-3].

The mammalian X and Y chromosomes evolved from an ancestral pair of autosomes. Divergence began when a mutation in SRY-box transcription factor 3 (*SOX3*) gave rise to the sex-determining region-Y (*SRY*) gene[4,5]. Male-specific inheritance exposed the proto-Y to unique evolutionary forces, facilitating and accumulation of the male-specific mutations in the vicinity of *SRY*[6]. This linkage of *SRY* and male-specific loci created selection for recombination suppression between the proto-X and proto-Y chromosome. Inversions are known to locally suppress recombination in heterozygotes[7,8]. Eventually, multiple independent inversions on the Y chromosome reinforced and expanded the regions of meiotic-recombination arrest, forming distinct evolutionary strata[9,10]. These strata expanded the non-recombining male-specific region, leaving the distal recombining portions known as pseudoautosomal regions. The human Y

chromosome contains five strata, three of which are shared across all eutherian mammals[10-13].

Recombination suppression led to the accumulation of deleterious mutations and repetitive DNA on the Y chromosome[14]. As a mechanism to maintain male fitness, large deleterious and non-functional DNA regions were deleted within each non-recombining stratum[14,15]. Although the Y chromosome experienced rapid early degeneration, this process slowed dramatically once essential genes had been purged, and the remaining genes have since remained stable[9,10,16,17]. Consequently, the human X chromosome retains approximately 98% of the ancestral autosome genes[18], whereas the human Y chromosome retains only approximately 3%[9]. Because of its sex-limited inheritance, the Y chromosome is subject to strong sex-specific selective forces and is therefore predicted to play an important role in sex fitness traits[14,19,20]. Two major evolutionary strategies are thought to preserve a specific set of Y genes: (1) maintenance of dosage-sensitive X-Y gene pairs, and (2) retention or amplification of testis-biased gene families[9,21].

The avian Z and W chromosomes, like the mammalian Y chromosome, evolved from an ancestral autosome pair. A series of events by inversions on the sex chromosomes suppressed crossing-over between the sex chromosomes, leading to the formation of evolutionary strata[22-24]. A Z-linked inversion suppressing recombination between the Z

and W chromosomes may have been selected by the accumulation of sexually antagonistic alleles nearby *doublesex* and *mab-3* related transcription factor 1 (*DMRT1*)[22]. Birds show a wide range of W chromosome degradation: from highly degraded in chickens to nearly homomorphic in ratites such as the emu[25,26]. Comparative genome analysis indicate that long-term recombination suppression has greatly reduced gene density on the W chromosome[22]. In chickens, the Z chromosome retains approximately 98% of the ancestral autosomal genes[3], whereas the W chromosome has lost most ancestral genes and now contains only a limited number of functional genes[27]. If a similar evolutional strategy of the Y chromosome should also operate on genes on the W chromosome, the W chromosome is expected to accumulate genes that are expressed in female-specific tissues[22,28]. However, the chicken W chromosome has no genes exclusively expressed in female sex-specific tissues[29]. Instead, the remaining W-linked genes are largely dosage-sensitive genes derived from ancestral autosomes[29,30]. The strong maintenance of these genes even in non-recombining regions is considered important evidence of the shared evolutionary pressures acting on the mammalian Y chromosome and the avian W chromosome[22,29].

I here hypothesized that dosage-sensitive W-linked genes may have acquired female-specific functions and used Japanese quail (*Coturnix japonica*) for experimental

model in this study. Comprehensive analysis of W-linked genes has not identified any functions related to female reproduction[29]. The Japanese quail, a member of the Phasianidae family, serves as a valuable experimental model and an alternative to the chicken due to its smaller body size, early sexual maturity, and shorter generation time[31-35]. The Hamburger and Hamilton (HH) stage is used for classification during the embryonic development of birds[36,37]. Sex determination and sex differentiation, which are important events in gonadal development, occur at HH25 and HH31, respectively. In my laboratory, RNA-seq analysis was performed to identify the W-linked genes expressed in embryonic gonads from just after sex determination to differentiation into testis and ovary in Japanese quail[38]. Ubiquitin associated protein 2 W-linked (*UBAP2W*) was identified as a W-linked gene significantly expressed in female gonads of Japanese quail at sex determination and morphological differentiation. Additionally, previous studies reported that *UBAP2* is strongly expressed in the ovaries[39,40]. Therefore, this study aimed to clarify whether the *UBAP2W* has acquired female-specific roles. In Chapter I, I verified the functional differentiation of *UBAP2W* and its Z-linked counterpart *UBAP2Z* using *in silico* analyses and verified these predictions through *in vivo* mRNA expression profile. In Chapter II, I performed UBAP2Z and UBAP2W protein expression analysis in the gonads using monoclonal antibodies.

Chapter I

Molecular divergences between the Z- and W-linked *UBAP2* alleles

1.1 Abstract

In birds, *UBAP2* is located on the sex chromosomes, with *UBAP2Z* on the Z chromosome and *UBAP2W* on the W chromosome. *UBAP2W* is significantly expressed in female embryonic gonads of the Japanese quail at sex determination and beginning morphological differentiation. However, the molecular differences between *UBAP2Z* and *UBAP2W* in Japanese quail has not been known well. To reveal their molecular divergence in Japanese quail, I performed *in silico* analysis using RNA-seq data of embryonic gonads of Japanese quail obtained in a previous study. The predicted *UBAP2Z* and *UBAP2W* cDNA sequences were 4,364 bp and 3,491 bp in length, respectively. The predicted amino acid sequences of UBAP2Z and UBAP2W were 1,113 AA and 1,104 AA, respectively. The identity of cDNA and amino acids sequences between them was 88.53 % and 86.39 %, respectively. The TPM-based expression analysis revealed distinct expression pattern between the two genes. Phylogenetic analysis of UBAP2 amino acid sequences indicated that UBAP2 differentiation occurred within orders. Finally, structural predictions suggested no detectable differences between UBAP2Z and UBAP2W at the protein level.

1.2 Introduction

The human Y chromosome currently harbors approximately 100 protein-coding genes[41]. Among these, seventeen genes have homologs on the X chromosome[9]. These genes can be classified into dosage-sensitive genes and genes that have acquired male-specific functions. Since sex chromosomes originally evolved from a pair of ancestral autosomes, loss of function of Y-linked genes can lead to dosage imbalance between X-Y pairs. To compensate for this, mammals maintain dosage balance via X chromosome inactivation in females[42,43]. However, some X-linked genes in X-Y escape inactivation[9], highlighting the importance of Y-linked gene expression in maintaining gene dosage. Consequently, dosage-sensitive Y-linked genes have been subject to strong selective pressure for their retention. In contrast, testis-specific genes have undergone massive amplification, forming tandem and palindromic repeats[44,45]. This amplification on the Y chromosome is considered an adaptive trait favored by natural selection[46]. Among the 17 surviving ancestral Y-linked genes, some genes have diverged functionally from their X homologs, acquiring male-specific roles in reproductive development or gametogenesis[47-49].

In chickens, the W chromosome contains approximately 29 protein-coding genes[27], all of which have homologs on the Z chromosome[50,51]. The W-linked genes show high sequence homology with their Z-linked counterparts[29]. Previous studies indicate that Z–

W pairs are enriched for haploinsufficient genes and maintain ancestral expression levels of essential genes, with the total expression of Z-W pairs in female comparable to the expression of two Z-linked gene copies in males[50,51]. Furthermore, the W-linked genes are expressed not only in sex-specific tissue but also in various other tissues[29,51]. The results that W-linked genes exhibiting high homology with Z alleles compensate for Z alleles expression in female and are ubiquitously expressed suggest that W-linked genes help maintain ancestral gene expression in birds without dosage compensation. However, while the female-specific function of W-linked genes have not been excluded, they remain insufficiently characterized.

In mammals, *UBAP2* is located on an autosome (chromosome 9 in humans and chromosome 4 in mice). The diverse functions have been reported, including regulation of KRAS proto-oncogene, GTPase (KRAS) activation and macropinocytosis in pancreatic cancer[52], maintenance of bone homeostasis through modulation of osteoblast and osteoclast differentiation[53], suppression of hepatocellular carcinoma cell invasion via ubiquitin-mediated degradation of Annexin A2[54], induction of phase separation and purinosome assembly in response to phosphoribosylaminoimidazole carboxylase and phosphoribosylaminoimidazolesuccinocarboxamide synthetase (PAICS) ubiquitination[55], regulation of UV-induced ubiquitination of RNA polymerase II as a human ortholog of

yeast RNAPII degradation factor 1 (Def1)[56], and promotion of radioresistance in hepatocellular carcinoma by enhancing homologous recombination through ubiquitination of solute carrier family 27 member 5 (SLC27A5)[57]. In Drosophila, Lingerer (lig), a known orthologue of human *UBAP2*, acts as a growth suppressor and has been shown to interact with the RNA-binding proteins Fragile X Mental Retardation Protein 1 (FMR1), Caprin (Capr), and Rasputin (Rin)[58]. *UBAP2* in mammals is predicted to be involved in protein degradation through ubiquitin-proteasome pathway. However, the functions of *UBAP2Z* and *UBAP2W* in birds has not been known well.

In this Chapter, I investigated the divergence between *UBAP2Z* and *UBAP2W* in Japanese quail. Due to incomplete genome information of Japanese quail, particularly for the W chromosome, *UBAP2Z* and *UBAP2W* sequences were predicted *in silico* using RNA-seq data previously reported and determined by sanger sequencing. After that, TPM values of *UBAP2Z* and *UBAP2W* were calculated by bowtie2-RSEM, and mRNA expression was further evaluated by RT-PCR and whole-mount *in situ* hybridization (WISH). Comparative analysis of amino acid sequences was conducted among Japanese quail, human, mouse and some other birds. Finally, the functions and protein structures of UBAP2Z and UBAP2W were predicted using InterPRO and AlphaFold3 based on their amino acid sequences.

1.3 Materials and methods

Prediction of UBAP2Z and UBAP2W sequences

The sequence of *UBAP2W* is unknown due to incomplete genome information on Japanese quail. Therefore, I predicted the sequence by *in silico* analysis using the RNA-seq dataset of the embryonic gonads from Japanese quail, accession number: PRJDB7305, using the method reported in our previous study[59]. The prediction method followed a previous study[60]. The male contigs were generated using RNA-seq data of male gonads and Trinity v2.14.0[61]. The female RNA-seq reads were aligned with male contigs by HISAT2 v2.2.1[62], and then the unmapped reads were extracted. The female-specific contigs were generated from the unmapped reads by Trinity and merged with the male contigs. Finally, each contig was annotated using chicken data (GRCg7b) and blastn v2.12.0[63], and the nucleotide sequences of *UBAP2Z* and *UBAP2W* were predicted.

Calculation of the expression levels by TPM

To examine the expression levels of the genes, TPM values were calculated using the RNA-seq data set mentioned above by bowtie2 v2.4.5[64] and RSEM v1.3.1[65] with contigs as a reference.

Animals

Fertilized Japanese quail eggs were purchased from Motoki Corporation (Saitama, Japan). Japanese quail were bred under 14L-10D light cycle (lighting on at 05:00 and off at 19:00) and were provided with food and water *ad libitum*. Females were mated and fertilized eggs were collected regularly. Fertilized eggs were incubated at 37.5°C. Genomic DNA was extracted from tails using lysis buffer containing 20 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 0.1% SDS and 1μg/ml Proteinase K at 55ºC overnight. After then, purification was performed by Phenol/Chloroform/isoamyl Alcohol (25:24:1). The sex of each embryo was determined by PCR genotyping using genomic DNA as the template according to the method described in a previous study[66]. The developmental stage of each embryo was determined based on the classification described in previous studies[36,37].

RNA extraction, cDNA synthesis, cloning and RNA probe synthesis

Total mRNAs were extracted from embryonic tissues of Japanese quail at HH27 using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. mRNA was reverse transcribed using the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA).

Primer sets were designed based on the predicted sequences *in silico* to amplify the coding sequence (CDS) of *UBAP2Z* and *UBAP2W* (Table 1). The amplified product was

cloned into the pUC19 vector and its sequence was determined using the SuperDye Cycle Sequence Kit v3.1 (Edge BioSystems, San Jose, CA, USA) and the ABI PRISM 3100 genetic analyzer (Applied Biosystems, Waltham, MA, USA).

The partial length sequence of *UBAP2Z* coding sequence was amplified using primer sets (Table 1) and KAPA Taq (NIPPON genetics). The amplified product was subcloned into pGEM-T Easy Vector Systems (Promega, Madison, WI, USA). The sequence of insertion was validated using SuperDye^{TM} Cycle Sequence Kit v3.1 (Edge BioSystems, San Jose, CA, USA) by ABI PRISM 3100 genetic analyzer (Applied Biosystems, Waltham, MA, USA). Antisense and sense RNA probe was transcribed from constructed plasmids using Digoxigenin RNA labeling Mix (Roche, Basel, Switzerland) and MAXIscript^{TM} SP6/T7 Transcription Kit (Thermo Fisher Scientific).

RT-PCR

The *UBAP2Z* and *UBAP2W* specific primers were designed (Table 1, Fig. 2). The PCR conditions were 94°C for 2 min, then 35 cycles of 94°C for 20 sec, 55°C for 20 sec, 72°C for 2 min. Amplified products were subjected to electrophoresis for 25 min in 1.5% agarose gel containing Midori Green Advance (NIPPON genetics, Tokyo, Japan).

WISH

Samples were dissected and fixed overnight with 4% PFA in PBS at 4°C. Samples were dehydrated in ethanol series and stored until use. Tissues were rehydrated with PBST before digestion in 1μg/ml Proteinase K for 10 min at 37℃. Tissues were washed two times in PBST, refixed for 10 min, and incubated for 1 hr at 65℃ in prehybridization buffer containing 50% formamide, 5 × SSC, 0.2% blocking reagent (Roche), 0.1% Triton-X, 0.1% CHAPS, 5 μg/ml yeast tRNA and 5 mM EDTA. The construct of RNA probes was described above. Tissues were incubated overnight at 65℃ in hybridization buffer containing antisense and sense Digoxigenin-labeled RNA probes, respectively. Following low and high stringency washes with 5 × SSC and 0.2 × SSC at 65°C, tissues were washed two times in KTBT, blocked for 1 hr at 37°C in blocking solution containing 1.5% blocking reagent in KTBT, incubated for 1 hr at 37°C in blocking solution containing Anti-DIG-AP, Fab fragments (1:2000; Roche). They were washed five times for 30 min in KTBT, and incubated overnight at 4°C in KTBT. Tissues were exposed to AP buffer containing NBT/BCIP for 5 hr at 37°C. After tissues were washed ten times for 10 min in PBST, dehydrated and rehydrated in ethanol series, samples were stored in 50% Glycerol/PBST and images were captured using a digital camera (DFC7000 T; LEICA,

Wetzlar, Germany) mounted on a microscope (M165 FC; LEICA).

Comparison of UBAP2 amino acids sequences

Phylogenetic tree analysis was performed to compare the UBAP2 amino acids sequences of *Coturnix japonica*, *Gallus gallus* (UBAP2Z: NP_001264034, UBAP2W: NP_001264033), *Lagopus muta* (UBAP2Z: XP_048788329, UBAP2W: XP_048786284), *Dromaius novaehollandiae* (UBAP2Z: XP_064358702, UBAP2W: XP_064356409), *Struthio camelus* (UBAP2Z: XP_068784443, UBAP2W; XP_068782123), *Turdus pilaris* (UBAP2Z: CAN4476373, UBAP2W: CAN4475872), *Taeniopygia guttata* (UBAP2Z: XP_012433062, UBAP2W: XP_072779166), *Chloris chloris* (UBAP2Z: CAN4663163, UBAP2W: CAN4663258), *Sylvia atricapilla* (UBAP2Z: XP_066195280, UBAP2W: XP_066194267), *Columba livia* (UBAP2Z: XP_064903099, UBAP2W: XP_064901133), *Alligator mississippiensis* (XP_059581304), *Anolis carolinensis* (XP_062828687), human (NP_001356988), and mouse (NP_081148). Multiple alignment was carried out using Clustal omega v1.2.4[67]. A phylogenetic tree based on the multiple alignment was constructed using the Maximum Likelihood method[68].

Prediction of the functions and structures of UBAP2Z and UBAP2W

The functions of UBAP2Z and UBAP2W were predicted from amino acid sequences by performing functional annotation using InterPRO[69]. To explore the differences of protein structure between UBAP2Z and UBAP2W, I performed AlphaFold3[70]. The quality of predicted structure was evaluated by ipTM score which measures the accuracy of the entire structure[71, 72]. A per-atom confidence was represented by pLDDT which estimated on a 0-100 scale where a higher value indicates higher confidence.

1.4 Results

UBAP2Z and *UBAP2W* showed high sequence similarity and conserved UBA domain

The sizes of the predicted nucleotide sequences of *UBAP2Z* and *UBAP2W ,* including those of their 5'- and 3'-UTR, were 4,364 bp and 3,491 bp, respectively (Fig. 1A). The 5'-UTR lengths of *UBAP2Z* and *UBAP2W* were 153 bp and 85 bp, respectively. The 3'-UTR of *UBAP2Z* and *UBAP2W* was 869 bp and 91 bp, respectively. The cDNA sequences of CDS determined after cloning of *UBAP2Z* and *UBAP2W* were 3,342 bp and 3,315 bp, respectively (Fig. 2). The CDS sequences were completely identical to the predicted sequences of both genes, and the *UBAP2Z* sequence was also completely identical to the registered predicted sequence in NCBI (*UBAP2Z*, XM_015848269). These results support the accuracy of the sequence predictions. The nucleotide sequence identity between *UBAP2Z* and *UBAP2W* CDS was 88.53% (Table 2). The predicted amino acid sequences of UBAP2Z and UBAP2W were 1,113 and 1,104 amino acids, respectively (Fig. 1B and 3), and were 86.39% identical (Table 3). Although the N-terminal UBA domain and DUF3697 were conserved in both genes, two and one amino acid substitutions were observed in the UBA domain and DUF3697, respectively, between UBAP2Z and UBAP2W.

UBAP2Z and *UBAP2W* showed different expression patterns in embryonic gonads

I calculated the TPM values using RNA-seq data to compare the expression patterns of Z and W alleles of the *UBAP2* gene (Fig. 4). The expression pattern of *UBAP2Z* was similar in males and females, showing increases in expression from HH27 to HH31 and decreases from HH31 to HH38. However, the expression level was higher in males than that in females at all stages. *UBAP2W* expression was highest at HH27, but gradually decreased from HH27 to HH38 in females. The overall expression level of *UBAP2W* was lower than that of *UBAP2Z*.

UBAP2Z and *UBAP2W* were ubiquitously expressed in embryonic tissues

The cloned sequences of UBAP2Z and UBAP2W analyzed by Sanger sequencing were consistent with the predicted sequences, respectively. Subsequently, I performed RT-PCR using six kinds of embryonic tissues at HH27 and WISH using gonads at HH27, HH31, HH34 and HH38 to confirm the mRNA expression of *UBAP2Z* and *UBAP2W*. *UBAP2Z* was expressed in six kinds of tissues of both sexes (Fig. 5). On the other hand, *UBAP2W* was expressed in six kinds of tissues in female. In addition, *UBAP2* mRNA was detected

in gonads of both sexes at HH27, HH31, HH34 and HH38 by WISH (Fig. 6). These results suggest that *UBAP2Z* and *UBAP2W* were not exclusively expressed in gonads but are expressed in ubiquitous tissues and are constitutively expressed in gonads.

Evolutional divergence between *UBAP2Z* and *UBAP2W* was shown

I examined the evolutional divergence of *UBAP2* among mammals, reptiles and birds using a phylogenetic tree (Fig. 7). *UBAP2* from birds clustered separately from *UBAP2* of mammals and reptiles. Within avian clusters, they were classified into the orders Paleognathae, Neoaves and Galloanserae. Basically, *UBAP2Z* and *UBAP2W* were classified by the orders. Functional differentiation of UBAP2W was not observed in Paleognathae but was observed in Galloanserae and Neoaves. In addition, the branch length of *UBAP2W* was longer than that of *UBAP2Z*, indicating a higher degree of amino acid substitutions in *UBAP2W*.

The predicted function and structure of UBAP2Z and UBAP2W were similar

InterPRO is program to predict protein domain and motifs using databases associated with protein family, domain and motif. The function of UBAP2Z and UBAP2W were

predicted using this. Both UBAP2Z and UBAP2W were defined as the same GO terms (Biological process: None, Molecular function: Protein binding, Cellular component: Cytoplasm, Nucleus). The predicted structures of UBAP2Z and UBAP2W by AlphaFold3 showed low quality overall (ipTM=0.17, respectively) (Fig. 8). Therefore, the comparison of whole structures between UBAP2Z and UBAP2W could not be performed. UBA domain of UBAP2Z and UBAP2W showed high quality (ipTM=0.76, respectively) and the difference between UBAP2Z and UBAP2W was none. Taken together, the divergence about protein structures between UBAP2Z and UBAP2W was not identified by *in silico* analysis.

1.5 Discussion

In non-model animals with limited genome information, *de novo* assembly is a commonly used method to generate contigs. In this study, the *UBAP2W* sequence was predicted using *de novo* assembly. The predicted *UBAP2Z* and *UBAP2W* sequences were consistent with the cloned cDNA sequences confirmed by Sanger sequencing, demonstrating that this method can accurately distinguish and predict highly homologous alleles. To determine the precise lengths of the 5'- and 3'-UTR of *UBAP2Z* and *UBAP2W*, RACE must be performed. However, the predicted those sequences showed differences. Although the CDS of *UBAP2W* showed high identity with *UBAP2Z*, the 5'- and 3'-UTR of *UBAP2W* were shorter than those of *UBAP2Z*. The 5'-UTR, a regulatory region at the beginning of a mRNA molecule, plays a crucial role in translation regulation and influences protein expression level[73]. Previous studies have highlighted the importance of 5'-UTRs in tightly regulating protein levels, particularly dosage-sensitive gene[74]. Therefore, the shorter 5'-UTR of *UBAP2W* may allow protein with less complex regulation.

The 3'-UTR regulates mRNA processes such as localization, stability, and translation. miRNAs modulate the gene expression at the post-transcription levels in many eukaryotic cells[75]. miRNAs recognize target sites in their 3'-UTR via base pairing, leading to their degradation or inhibition of their translation[76-78]. Genes with longer 3'-

UTRs are regulated by more distinct types of miRNAs[79]. Consequently, the shorter 3'-UTR of *UBAP2W* compared *UBAP2Z* may reduce susceptible to post-transcriptional regulation via miRNAs. Overall, the shortened 5'-and 3'-UTRs may stabilize protein expression by simplifying post-transcriptional regulations.

Previous reports indicate that Z- and W-linked gene pairs are broadly expressed, and that the total expression of Z- and W-linked genes in females is equivalent to that of the two Z-linked copies in males[29,32]. Consistent with this, *in vivo* analysis by RT-PCR and WISH in this study showed that *UBAP2Z* and *UBAP2W* mRNAs are ubiquitously expressed in embryonic tissues and are constantly expressed in the gonads from HH27 to HH38. However, their expression dynamics differ: *UBAP2W* mRNA expression decreased from HH27 to HH38, unlike *UBAP2Z*. This suggests that *UBAP2Z* and *UBAP2W* are regulated by distinct mechanisms during gonadal development and may have divergent functions.

The amino acid sequences between UBAP2Z and UBAP2W showed high identity, similar nucleotide sequences. Interestingly, UBAP2Z exhibits higher sequence identity to mammalian UBAP2 than to UBAP2W (Table 3). Considering that regions of the chicken Z chromosome are orthologous to mammalian autosomes[1,2,3], it is plausible that UBAP2Z functions in general cellular homeostasis similar to its mammalian counterpart. Both

UBAP2Z and UBAP2W conserve the UBA domain and DUF3697 at the N-terminal region, suggesting roles in protein binding. The UBA domain is a ubiquitin-binding domain that binds polyubiquitin substrates[80], and it plays a critical role in protein degradation through the ubiquitin–proteasome system. In Japanese quail, the predicted amino acid sequences of UBAP2Z and UBAP2W exhibit high homology and both contain the UBA domain, suggesting that they may participate in the ubiquitin–proteasome pathway. The ubiquitin-proteasome pathway is strictly regulated by ubiquitin ligase E1, E2 and E3. E3 ligase selectively recognizes target proteins and transfers ubiquitin from E2 ligase to the target proteins. E3 ligases are classified into Homologous to E6-AP C terminus (HECT) type contained HECT domain, RING-finger type contained RING domain, U-box type contained U-box domain and RING-IBR-RING (RBR) type contained a RING1, a central in-between-RINGs and a RING2 domain [81-83]. UBAP2Z and UBAP2W did not contain HECT and RING domain. Therefore, UBAP2Z and UBAP2W do not act as E3 ligase and may play as coactivator of ubiquitin-proteasome pathway. DUF3697 is a domain of unknown function, but it has been suggested that DUF3697 in Ubiquitin associated protein 2 like (UBAP2L), a homolog of UBAP2, enhances binding with interacting proteins[84]. DUF3697 in UBAP2Z and UBAP2W is expected to play a similar role.

Phylogenetic analysis of UBAP2 revealed three avian clusters corresponding to Paleognathae, Neoaves and Galloanserae, alongside mammalian and reptilian clusters. This pattern suggests that the UBAP2 differentiation occurred before the divergence of these avian lineages. The W chromosome of Paleognathae is not highly differentiated from the Z chromosome[25,26], accordingly UBAP2Z and UBAP2W of emu and ostrich cluster together, suggesting that UBAP2W retains functions similar to UBAP2Z, like an ancestral autosome. In contrast, UBAP2W of Galloanserae and Neoaves showed lineage-specific divergence from UBAP2Z, with Galloanserae UBAP2W exhibiting differentiation comparable to that of Neoaves. UBAP2W is suggested to show evolutionary divergence from UBAP2Z in Neognathia, including Japanese quail.

Protein structure prediction using AlphaFold3 was challenging due to the reliance on existing structural data, making it difficult to model proteins with low homology or intrinsically disordered regions. It has been suggested that mammalian UBAP2 possesses numerous intrinsically disordered regions[55]. This thought to be the reason why the whole structure of UBAP2Z and UBAP2W could not be predicted. While the predicted UBA domain structures of UBAP2Z and UBAP2W were high quality, no structural differences were observed between the two proteins. Therefore, UBAP2Z and UBAP2W may have the ability to bind to ubiquitin chains.

1.6 Tables

Table 1 Primers used in this chapter

Primer name	Experiment	Sequence 5' to 3'
2550F	sex check	GTTACTGATTCGTCTAGGAGA
2718R		ATTGAAATGATCCAGTGCTTG
UBAP2_F_5'UTR	Cloning	TGTATATGATGACTTCGGTG
UBAP2Z_R_stop		TTAGTTTGTCCAGTATGCAG
UBAP2W_F_5'UTR	Cloning	GGCTGGCTTCTTTCG
UBAP2W_R_stop		TTAGTTAGTCCAGTATGGAG
GAPDH RT-SE re.	RT-PCR	TGTGACTTCAATGGTTACAG
GAPDH RT-AS re.		CAGATCAGTTTCTATCAGCC
UBAP2_F1	RT-PCR	GACAGAGAAGYGAKTCGTG
UBAP2_R7		TTGAAGACAACACTGTGGCAG
UBAP2_F_5'UTR	RT-PCR	TGTATATGATGACTTCGGTG
UBAP2_R3		GTTGGTTCAGATGGAGCC
UBAP2_F3	WISH	GGCTGCCAATGGATTACTATG
UBAP2_R4		TTAGTTTGTCCAGTATGCAG
M13 Forward	Sequence	GTAAAACGACGGCCAGT
M13 Reverse		CAGGAAACAGCTATGAC
UBAP2_R1		CCACGTCCAAAYCCTCTC
UBAP2_R2		TAAGTCATCATAGCCTGCTC
UBAP2_R3		GTTGGTTCAGATGGAGCC
UBAP2_F2		CCTTTGAATACCTCTTTGCC
UBAP2_seqF		AAGAGACAGAAGGCAACTC
UBAP2_seqR		CCCATACTGGAATGCAC

Table 2 Identity of nucleotide sequences

	UBAP2Z Quail	UBAP2W Quail	UBAP2Z Chicken	UBAP2W Chicken	UBAP2 Human	UBAP2 Mouse
UBAP2Z Quail		88.53	95.07	88.78	72.57	71.79
UBAP2W Quail			89.71	94.78	71.52	70.33
UBAP2Z Chicken				90.55	74.09	72.51
UBAP2W Chicken					72.35	70.52
UBAP2 Human						83.24
UBAP2 Mouse						

Table 3 Identity of amino acid sequences

	UBAP2Z Quail	UBAP2W Quail	UBAP2Z Chicken	UBAP2W Chicken	UBAP2 Human	UBAP2 Mouse
UBAP2Z Quail		86.39	95.07	86.68	70.47	67.42
UBAP2W Quail			85.59	92.97	66.20	64.63
UBAP2Z Chicken				88.50	72.11	68.44
UBAP2W Chicken					67.90	65.16
UBAP2 Human						79.35
UBAP2 Mouse						

1.7 Figures

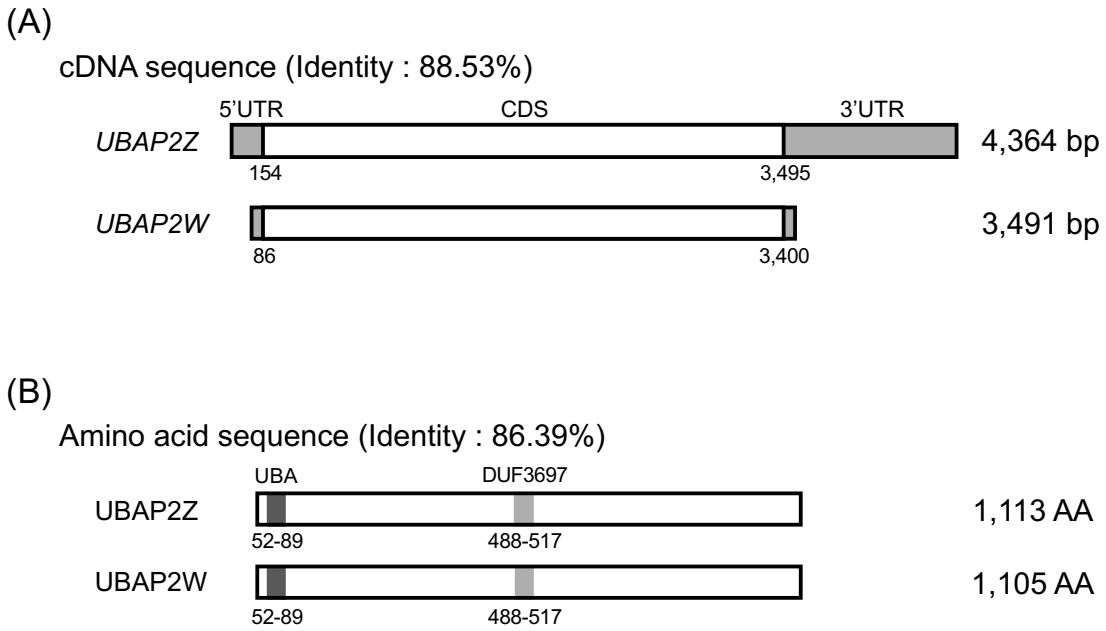

Fig. 1 Predicted cDNA and amino acid sequences of *UBAP2Z* and *UBAP2W*

(A) cDNA sequences of *UBAP2Z* and *UBAP2W* were predicted from generating contigs. (B) Amino acid sequences of UBAP2Z and UBAP2W were predicted from cDNA sequences of *UBAP2Z* and *UBAP2W*, respectively.

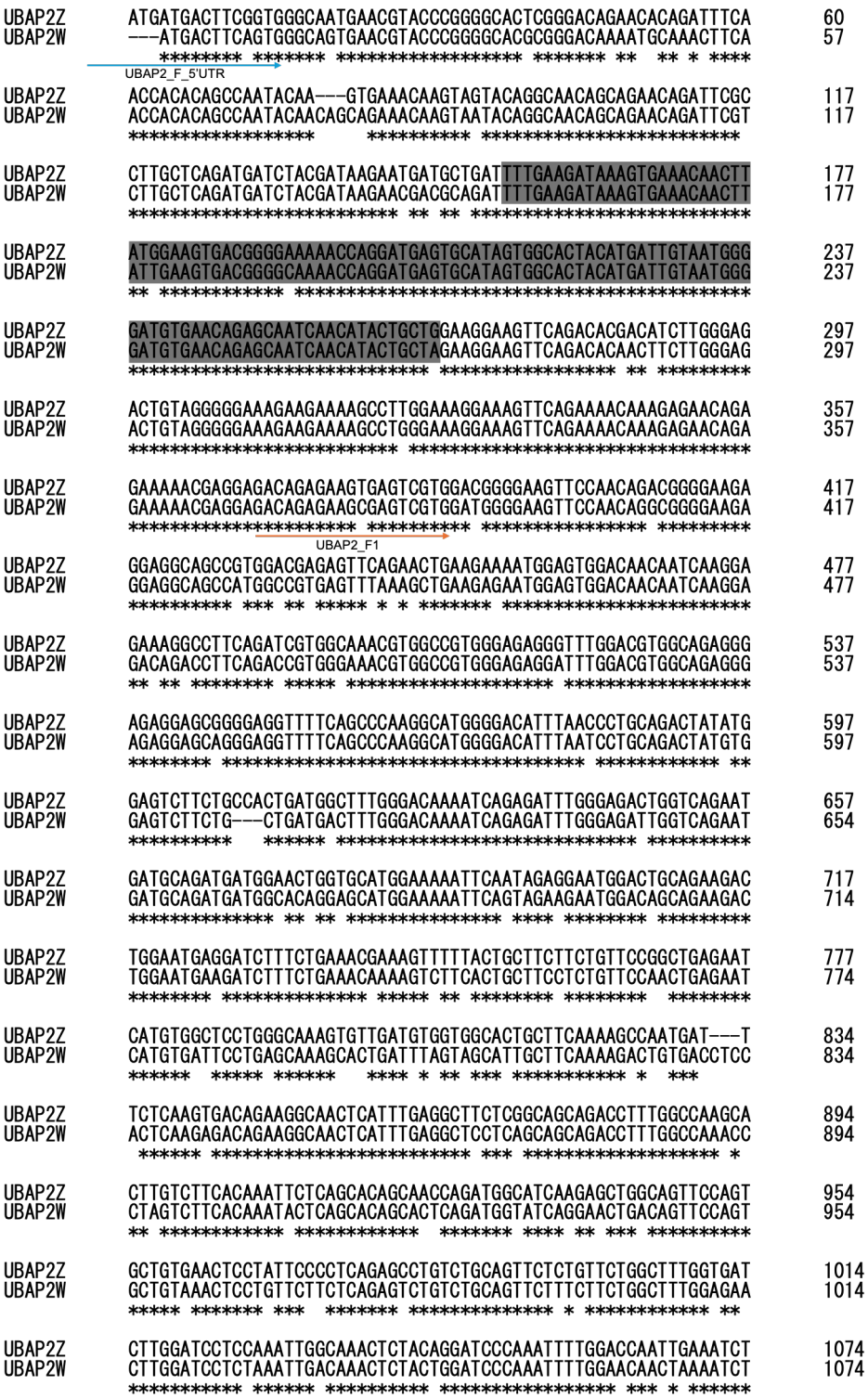

Fig. 2 The coding region sequences of *UBAP2Z* and *UBAP2W*

Alignment of CDS sequences between *UBAP2Z* and *UBAP2W*. Dark grey and light grey indicate UBA domain and DUF3697, respectively. Blue arrow and orange arrow indicate UBAP2Z specific primer set and UBAP2W specific primer set, respectively.

Fig. 2 The coding region sequences of *UBAP2Z* and *UBAP2W* (continued)

```
UBAP2Z      ACAAACAGCCTTGGCCTGAGCGTTACCAGTGTGTCTATGCCAGCTGCCACCACAAGGGCA      2325
UBAP2W      ACAAACAGCCTTGGCCTGAGTGTTACCAGTGTGTCTATACCAGCTGCTACTGCACGAGCA      2298
            ********************* ***************** ******** **  ** * ***

UBAP2Z      GCTCCCTTAGTGTCTTCAGGCAAAGCTCCCCCCAACCTGCCCCAGGGTGTACCGCCCTTG      2385
UBAP2W      GCTCCCTTATTGACTTCAGGCAAAGCTCCTCCAAACCTGCCCCAGGGTGTACCACCCTTG      2358
            ********* ** ***************** ** ******************** ******

UBAP2Z      TTGCATAACCAGTATATTGTGGGACCTGGAGGACTTCTACCTGCCTACCCAATTTATGGT      2445
UBAP2W      TTGCATAATCAGTATATTGTGGGACCTGGAGGACTTCTGCCTGCCTACCCAATTTATGGT      2418
            ******** ***************************** ********************

UBAP2Z      TATGATGATCTTCAGATGCTTCAATCCAGGCTGCCAATGGATTACTATGGAATTACATTC      2505
UBAP2W      TATGATGATCTCCAGATGCTTCCATCCAGGCTACCAATGGATTACTATGGAATTACATTC      2478
            *********** ********** ********* ***************************

UBAP2Z      CCTGCTCCTGCAACCTTGACAGGCAGAGATGGGAGCCTTGCTAACAACCCATACTCAGGT      2565
UBAP2W      CCCACTCCTGCAACCTTGACAGGCAGAGATGGAAGCCTTGCTAACAACCCATACTCAGGT      2538
            **  *************************** ***************************

UBAP2Z      GATGTAACAAAATTTGGCCGTGGGGATTCTGCCTCTCCTGCTCCTGCAACCACGCTAGCT      2625
UBAP2W      GATGTAACAAAATTTGGCCGTGGGGATTCTACCTCCCCTGCTCCTGCTACAACTATAGCC      2598
            ****************************** **** ********** ** **  ****

UBAP2Z      CAGCCACAGCAGAACCAGACACAGACTCACCACACAACTCAGCAGCCCTTCCTCAACCCA      2685
UBAP2W      CAGCAACAGCAGAACCAGACACAGACTCACCATACGACTCAGCAGCCCTTCCTCAACCCA      2658
            **** *************************** ** ************************

UBAP2Z      ACCCTGCCACCAGGCTACAGCTACACTGGGCTACCTTACTATGCTGGGGTTCCAGGTGTA      2745
UBAP2W      ACCCTGCCACCGGGGTACAGCTACACTGGGTTACCTTACTATGCTGGGGTGCCAGGCATA      2718
            *********** ** *************** ******************* *****  **

UBAP2Z      CCCAGTGCATTCCAGTATGGGCCAACCATGTTTGTACCTCCAGCATCAGCAAAGCAGCAT      2805
UBAP2W      CCCAGTGCATTCCAGTATGGGCCAACCATGTTTGTGCCTCCAGCATCTGCTAAACAGCAT      2778
            ********************************** ********** ** ** ******

UBAP2Z      GGAGTCAACCTGAACACTGCCTCGACTCCTTTCCAGCAAGCGAGTGGCTACGGGCAGCAT      2865
UBAP2W      GGAGTCAATTTGAACACTGCCTCGACTCCTTTCCAGCAAGCAAGTAGCTATGGGCAGCAT      2838
            ********  ******************************** *** **** *********

UBAP2Z      GGCTACGGAGCAGCAGGCTATGATGACTTAACGCAGGGAACGGCAGCTGGAGATTACAGC      2925
UBAP2W      GGCTATGGAGCAGCAGGCTATGATGACTTAACACAGGGAACAGCAGCTGGAGATTACAGC      2898
            ***** ************************** ******** ******************

UBAP2Z      AAAGGAGGCTACAGTGGGTCATCTCAAGCACAAAACAAATCTGCTGGCACTGGACCTGGG      2985
UBAP2W      AAAGGAGGCTACAGTGGGTCATCTCAAGCACAAAGCAAATCTGCTGGCACTGGACCTGGG      2958
            ********************************** ************************

UBAP2Z      AAAGGTGTTTCTGTGACCTCAAGTAACACAGGTGTGCCTGACATCAGTGGCTCAGTCTAT      3045
UBAP2W      AAAGGTGTTTCTGTAACCTCAAGTAACACAGGTGTGCCTGATATCAGTGGCTCAGTCTAT      3018
            ************** ************************* ******************

UBAP2Z      AACAAGACTCAGACGTTCGACAAGCAGGGATTCCATGCTGGCACACCACCTCCCTTCAGC      3105
UBAP2W      AACAAGACTCAGACTTTTGACAAGCAGGGATTCCATGCTGGCACACCACCTCCTTTCAGC      3078
            ************** ** ********************************** ******

UBAP2Z      CTGCCCTCTGCTCTTGGATCTACTGGGCCCCTGAACCCTGGTGCTGCCCCGGGTTACGCT      3165
UBAP2W      ATGCCCTCTGCTCTTGGATCTACTGGGCCCCTGAACCCTGGTGCTGCCCCAGGTTATGCT      3138
             ************************************************* ***** ***

UBAP2Z      CCAGCCCCTTTTCTCCACATCCTGCCTGCGCATCAGCAGCCTCACTCCCAGATGCTGCAT      3225
UBAP2W      CCAGCTCCTTTTCTTCATATTCTGCCTGCACATCAACAGCCTCATTCACAGATGCTGCAT      3198
            ***** ******** ** ** ******** ***** ******** ** ************

UBAP2Z      CACCACCTTCAGCAGGATGGGCAGGGAGGATCTGGACAGCGCAACCAGCCCAGCACCATG      3285
UBAP2W      C---ATCTTCAGCAAGATGGGCAGGGTGGATCTAGCCAGCGCAACCAGCCCAGCACCATG      3255
            *   * ******** *********** ****** * ************************

UBAP2Z      CAGCAAAAGTCTCAGGCTACCAAAACCGCCTACAGCACTTCTGCATACTGGACAAACTAA      3345
UBAP2W      CAGCAAAAATCTCAGGCTACCAAATCTGCCTATGGCACTTCTCCATACTGGACTAACTAA      3315
            ******** *************** * *****  ******** ********** ******
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Fig. 2 The coding region sequences of *UBAP2Z* and *UBAP2W* (continued)

Fig. 3 The amino acids sequences of UBAP2Z and UBAP2W

Alignment of the amino acid sequences between UBAP2Z and UBAP2W. Dark grey and light grey indicate UBA domain and DUF3697, respectively. Red arrow heads indicate amino acid substitutions between UBAP2Z and UBAP2W.

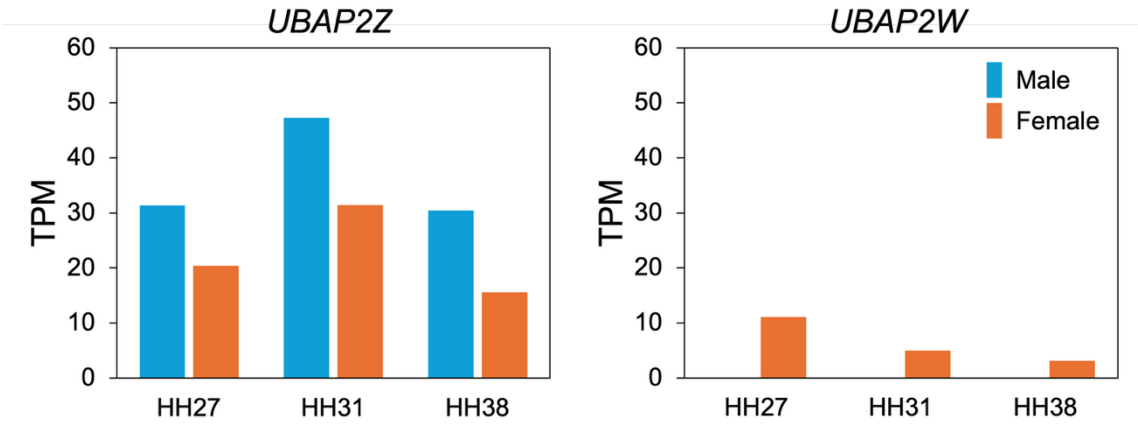

Fig. 4 The expression levels of *UBAP2Z* and *UBAP2W* in embryonic gonads of Japanese quail

Expression levels of *UBAP2Z* and *UBAP2W* measured by TPM. Light blue and orange bars indicate male and female, respectively.

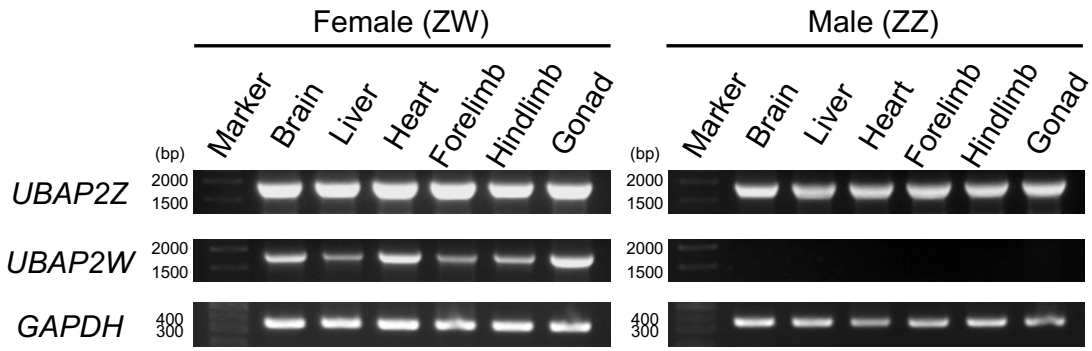

Fig. 5 RT-PCR using six kinds of embryonic tissues at HH27

The mRNA expression of *UBAP2Z* and *UBAP2W* in brain, heart, liver, forelimb, hindlimb, gonad at HH27 was evaluated by RT-PCR. *GAPDH* was used as a house keeping gene.

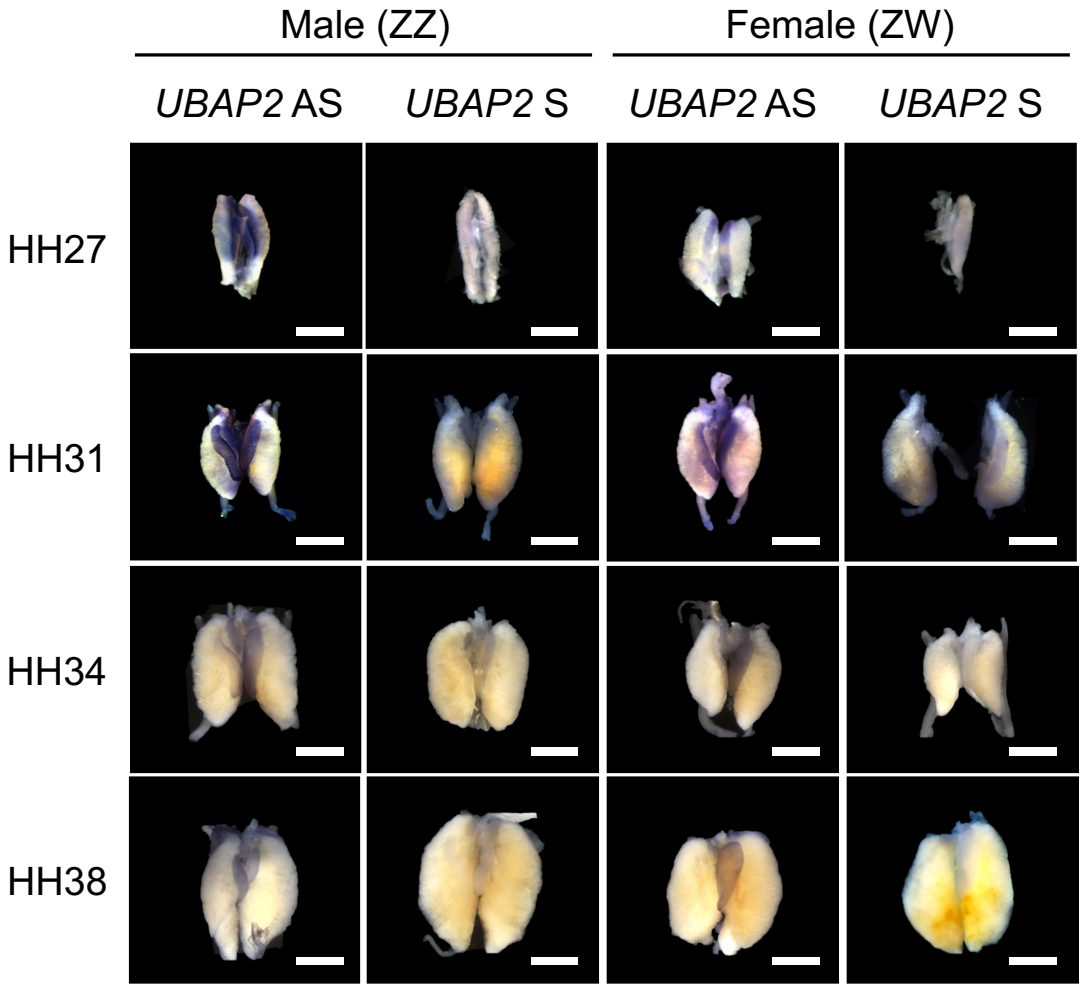

Fig. 6 WISH using gonads at HH27, HH31, HH34 and HH38

The *UBAP2* mRNA expression in gonads at HH27, HH31, HH34 and HH38 was evaluated by WISH. Scale bars indicate 1 mm. AS and S indicate Anti-sense and Sense, respectively.

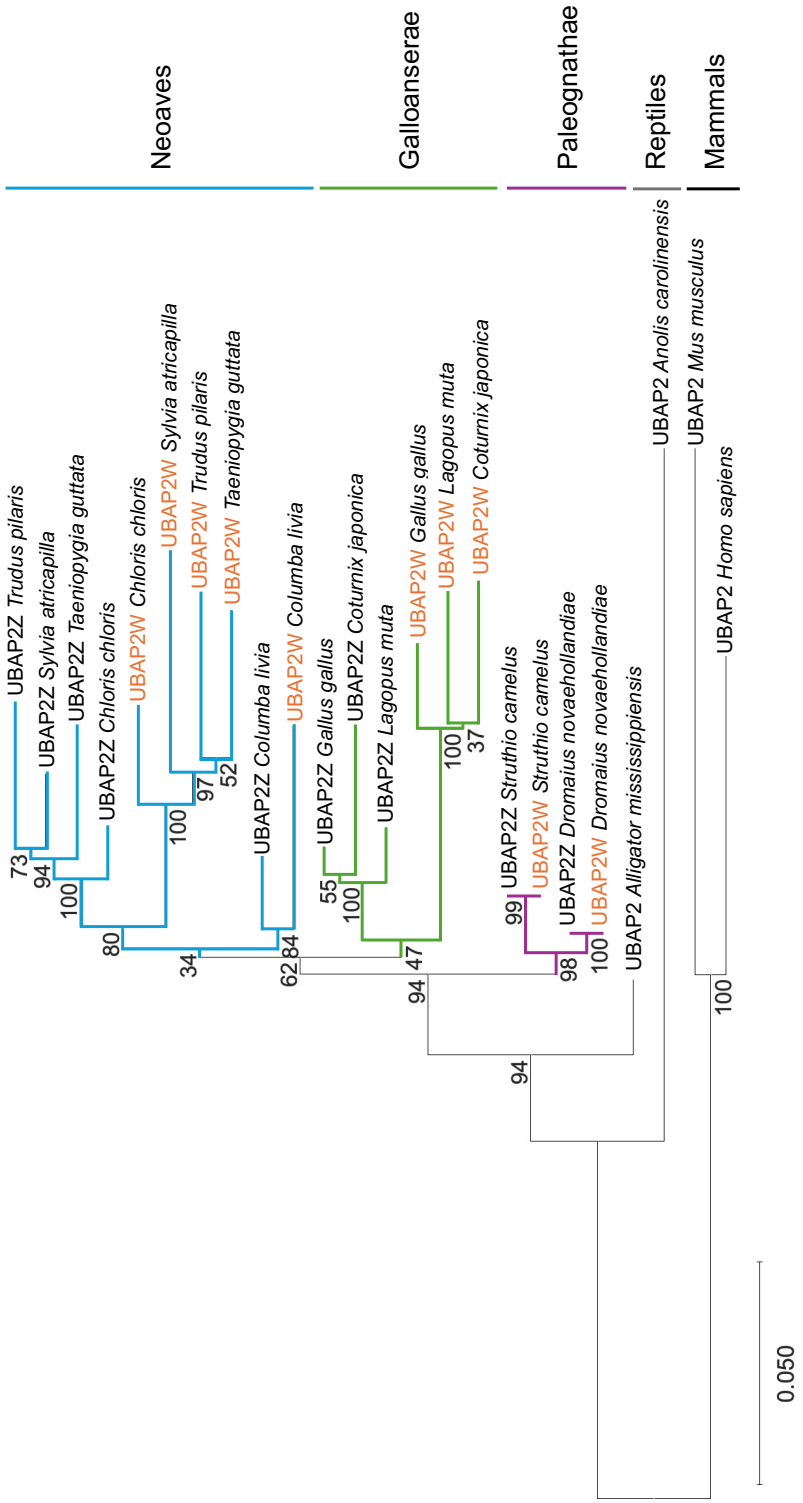

Fig. 7 Phylogenetic tree of UBAP2 amino acid sequences

The classification of UBAP2 was performed by phylogenetic tree using Maximum Likelihood method.

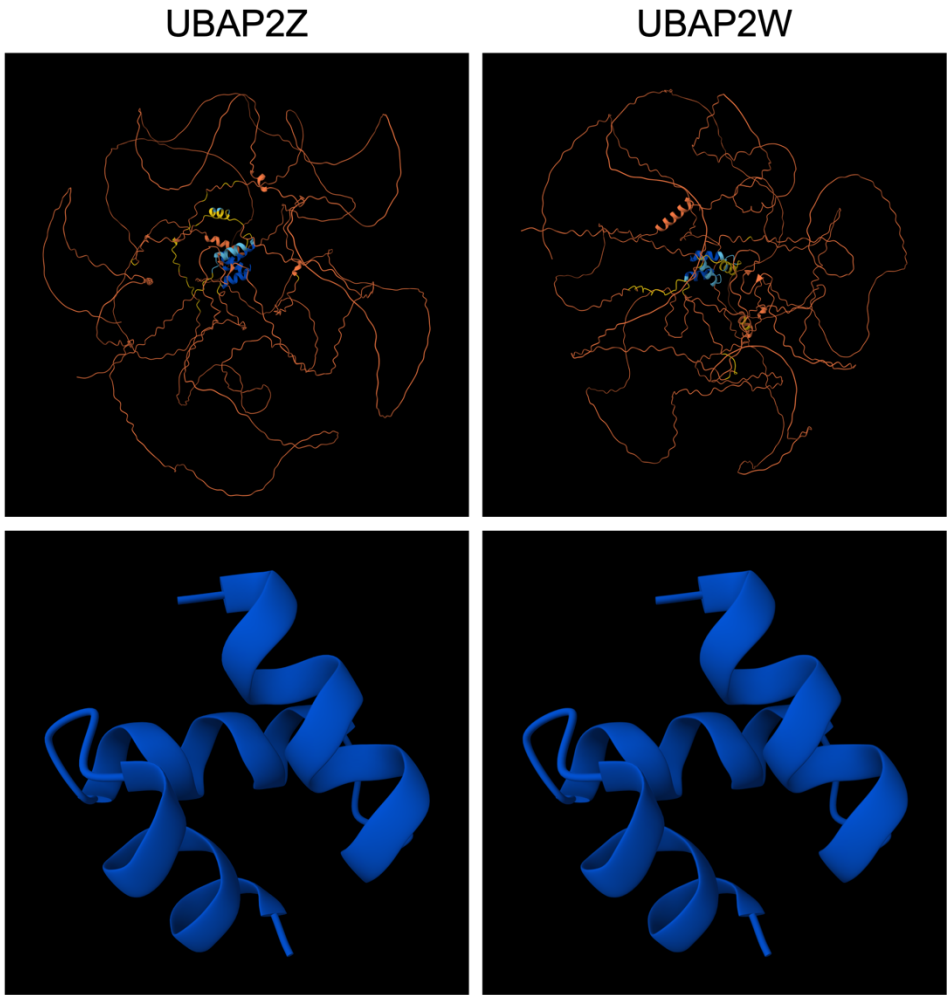

Fig. 8 Predicted protein structures of UBAP2Z and UBAP2W

Protein structures of UBAP2Z and UBAP2W were predicted from amino acid sequences by AlphaFold3. The top images showed UBAP2Z (left) and UBAP2W (right), and the bottom images showed UBA domain of UBAP2Z and UBAP2W. Blue, light blue, yellow and orange indicate very high (plDDT>90), confident (90>plDDT>70), low (70>plDDT>50) and very low (plDDT<50), respectively.

Chapter II

Expression dynamics of UBAP2Z and UBAP2W protein in embryonic gonads

2.1 Abstract

In mammals, Y-linked genes play key roles in the sex determination and spermatogenesis. By contrast, the functions of W-linked genes in birds remain largely unknown. The high homology between the Z and W alleles makes them difficult to distinguish and analyze. In fact, it is not even clear whether the proteins of W-linked genes are expressed. In this chapter, to reveal the divergence of UBAP2Z and UBAP2W proteins in embryonic gonads, I performed protein expression and protein-protein interaction analyses. First, monoclonal antibodies against UBAP2Z and UBAP2W were generated for western blotting and immunohistochemistry (IHC), and their specificity was validated using recombinant proteins. Protein expressions of UBAP2Z and UBAP2W were then examined by Western blotting. Second, to validate the protein-protein interactions, I performed far-western blotting using HH38 gonadal lysates and recombinant proteins. UBAP2W interacted with proteins expressed exclusively in female, whereas UBAP2Z interacted with proteins expressed in both sexes. Finally, I performed IHC using DDX4, a germ cell marker, and SOX9, a Sertoli cell marker. UBAP2Z was expressed in the germ cells and Sertoli cells in males from HH27 to HH38, and in females it was detected in germ cells at HH27 and HH31 and in somatic cells from HH27 to HH38. In contrast, UBAP2W was consistently expressed in female germ cells from HH27 to HH38, but

become to somatic cells from HH34 onward. In conclusion, it was revealed that UBAP2W interacted with different proteins than UBAP2Z and to exhibit different localization patterns from UBAP2Z in the gonads.

2.2 Introduction

Genes located on sex-specific sex chromosomes, the mammalian Y chromosome and the avian W chromosome, are thought to contribute to sexual development[20,85,86]. Among the approximately 100 protein-coding genes on the human Y chromosome[31], 17 have homologs on the X chromosome. For example, *SRY* evolved from its X-linked homologue, *SOX3*[87]. Although SRY and SOX3 are highly divergent at the amino acid level (approximately 44% identical), both proteins contain an DNA-binding domain, HMG domain, that is more conserved (approximately 71% identical)[88]. *SRY* is a male sex-determining gene to upregulate the SRY-box transcription factor 9 (*SOX9*) expression for testis differentiation[89]. Another example is the DEAD box helicase 3 X-linked (*DDX3X*) gene, which is widely expressed in body sexes, particularly in the brain, liver, and hematopoietic tissues. Its functions are diverse, including development, cell proliferation, and RNA metabolism[90-92]. The Y homolog *DDX3Y* shows 92% amino acid identity with *DDX3X* but is expressed specifically in the testis, where it primarily functions in spermatogenesis[93,94]. Despite their high similarity, *Ddx3y* cannot compensate for the loss of *Ddx3x* in embryonic development in mice[95]. DDX3X has higher catalytic activity than DDX3Y, likely due to the differences in ATP hydrolysis rates[96]. Together, these findings suggest functional divergence between DDX3X and its Y-linked DDX3Y.

In birds, the histidine triad nucleotide-binding protein W-linked (HINTW) which has a Z-linked homologue HINTZ, was once considered a candidate sex-determining gene[97,98]. Among the 29 protein-coding genes on the chicken W chromosome, HINTW and HINTZ show the greatest divergence, sharing approximately 65% amino acid identity[97]. HINT proteins generally have endogenous adenosine 5' monophosphoramidate enzyme activity. Chicken *HINTZ* retains the functional catalytic domain, the HIT motif, like other HINT proteins[97]. By contrast, this motif is absent in *HINTW* of chicken. Similarly, a nucleotide binding site confirmed in *HINTZ* was not conserved in *HINTW*. Several *in vitro* biochemical experiments showed that HINTZ function can be inhibited via the formation of HINTZ/HINTW heterodimers[99]. However, ZZ embryos overexpressing *HINTW* develop to normal males with bilateral testes[100]. This provides genetic evidence against a role for *HINTW* in avian sex determination. At present, the functions of other W-linked genes have not been known well, and no study has confirmed endogenous W chromosome gene protein, including HINTW.

Monoclonal antibodies are useful for distinguishing between highly homologous Z and W allele proteins. Monoclonal antibody production using the hybridoma technique was first established by Köhler and Milstein[101]. In this method, spleen cells from antigen-immunized mice are fused with unlimitedly growing myeloma cells to form B cell

hybridomas, which produce highly homogeneous monoclonal antibodies targeting the same epitope. The more efficient method for generating monoclonal antibody using rats was established than conventional method using spleen[102]. Therefore, this method was performed in this study. Compared with polyclonal antibodies, monoclonal antibodies have high purity, predefined specificity, good reproducibility, and can be supplied continuously[103]. Notably, monoclonal antibodies have the specificity to recognize just one amino acid difference in an antigen[104], making them powerful tools for precise molecular discrimination.

In this chapter, I aimed to reveal the divergence in protein expression between UBAP2Z and UBAP2W in the gonads. First, I performed to generate monoclonal antibodies against UBAP2Z and UBAP2W for Western blotting and IHC. All generating antibodies were screened using UBAP2Z and UBAP2W recombinant proteins by Western blotting and immunocytochemistry (ICC). Next, I performed Western blotting to evaluate the protein expression of UBAP2Z and UBAP2W in gonads. Then, to evaluate protein-protein interaction of UBAP2Z and UBAP2W, I performed far-western blotting. Finally, IHC using some cell type markers was performed to identify the localization of UBAP2Z and UBAP2W in gonads.

2.3 Materials and methods

Animals

I followed the description in Chapter I.

RNA extraction, cDNA synthesis and cloning

I followed the method in Chapter I. The CDS sequences of UBAP2Z and UBAP2W were amplified using primer sets (Table 4) and PrimeSTAR MAX ver.2 (Takara). The amplified products were cloned into pUC19 vector using In-Fusion cloning system (Takara). Restrict enzyme digestion was performed using *EcoR*I and *Xba*I, and the fragments were subcloned into pcDNA3.1 vector for expressing recombinant UBAP2Z and UBAP2W proteins.

Generation of monoclonal antibodies

The plasmids of UBAP2Z and UBAP2W for the generation of monoclonal antibodies were prepared by the method described in Chapter I and sent to Shigei Medical Research Institute. The monoclonal UBAP2Z and UBAP2W antibodies were produced by the iliac lymph node method[105] using UBAP2Z and UBAP2W recombinant proteins as antigens by Dr. Matsuyama and Dr. Kobayashi in Division of Molecular Genetics, Shigei Medical Research Institute. To obtain the numerous numbers of B cells, rats were used in this study.

Subsequently, the supernatants of hybridoma clones were screened using ELISA by Dr. Matsuyama and Dr. Kobayashi. The antibodies which showed positive reactions were sent to my laboratory. The supernatants of hybridoma clones were screened to determine antibody specificity and reactivity using ICC and western blotting with recombinant proteins expressed in COS7 cells. The supernatants of hybridoma clones (UBAP2Z #24-1, UBAP2W #2-6-7 and UBAP2W #2-29-3) showing the best signals with the lowest backgrounds were chosen.

Cell culture and Transfection

COS-7 cells were plated in a 60-mm dish at a density of 0.8×10^6 cells/dish and cultured in DMEM supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2. Following the manufacturer's protocol, COS7 cells were transfected with 5 μg pcDNA3.1-UBAP2Z or 5 μg pcDNA3.1-UBAP2W using Lipofectamine3000, to express UBAP2Z and UBAP2W recombinant proteins, respectively.

ICC

COS7 cells were subcultured on a coverslip and transfected with the UBAP2Z and UBAP2W expression plasmid, respectively. COS7 cells on a coverslip were fixed in 4% PFA for 10 min. The cells were permeabilized in 0.25% Triton X-100 for 5 min followed

by three washes in PBS and incubated with the supernatants of the hybridoma clones containing primary antibodies (anti-UBAP2Z antibody #24-1 or anti-UBAP2W antibody #2-29-3) at 4°C overnight. After washing the cells on coverslips with 0.05% tween 20 in PBS, they were incubated in goat anti-rat IgG conjugate Alexa 488 antibody (1:10000, Abcam, ab150157) at 37°C for 2 hr. After a further wash in 0.05% tween 20 in PBS and subsequent staining with Hoechst, the fluorescence of the cells on the coverslips was captured using a CCD camera (DS-Ri1; Nikon, Tokyo, Japan) mounted on a fluorescence microscope (ECLIPSE E800; Nikon).

Protein extraction

Proteins were extracted from embryonic gonads of males and females at HH27, HH31, HH34, and HH38. Gonads were pooled and homogenized in RIPA buffer (Merck, Darmstadt, Germany) containing 1 mmol/L sodium orthovanadate in ultrapure water, 2 mmol/L PMSF in DMSO, and protease inhibitor cocktail in DMSO (NAKALAI TESQUE, Kyoto, Japan). After rotation for 1 hr, debris was removed by centrifugation at 10,000 × g for 10 min at 4°C. The supernatants were used as protein extracts and preserved at -80°C until use. The protein concentration was measured by the BCA method.

Western blotting

Protein extracts (20 μg per lane) were resolved by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis at a constant current of 25 mA for 80 min. The resolved proteins were transferred at a constant current of 55 mA for 1 hr onto a PVDF membrane (pore size: 0.45 μm, Merck, Darmstadt, Germany) treated with methanol for 1 min. The membrane was incubated in 5% (w/v) skim milk in TBS-T for 1 hr. After blocking, the membrane was reacted with rat anti-UBAP2Z monoclonal antibody #24-1 or rat anti-UBAP2W monoclonal antibody #2-6-7 (1:100) in 5% skim milk in TBS-T at 4°C overnight and washed three times in TBS-T. The membrane was then reacted with goat anti-rat IgG-HRP (1:10000, Proteintech, SA00001-15) in TBS-T for 1 hr and washed three times in TBS-T. The signals were visualized using Immobilon (Merck) and were detected by LAS-3000 (FUJIFILM, Tokyo, Japan).

Far-western blotting

In addition to western blotting, far-western blotting was performed by blocking the blotted membranes with a five-fold dilution of N101 reagent (NOF, Tokyo, Japan) for 30 min and then incubating the membranes with 25 μg recombinant proteins extracted from COS-7 cells in 5 ml TBS-T for 1 hr.

IHC

Isolated gonads were fixed in 4% PFA, embedded in TissuePrep (Fisher Scientific, Waltham, MA, USA), and sectioned at a thickness of 5 μm. Sections were deparaffinized, rehydrated in ethanol series, and autoclaved at 2 atmospheres, 105°C for 15 min in 10 mM citric acid buffer. After blocking in PBS containing 10% normal goat serum and 0.1% (w/v) Triton X-100 for 30 min, the sections were incubated with a mixture of primary antibodies (rat anti-UBAP2Z monoclonal antibody #24-1 [1:250], rat UBAP2W monoclonal antibody #2-29-3 [1:250], rabbit anti-DDX4 antibody provided by associated professor Mizushima [1:250], and mouse anti-SOX9 antibody (Abnova, Taiwan, H00006662-A01) [1:250]) at 4°C overnight and washed three times with PBS. The samples were visualized after incubation with a mixture of secondary antibodies (goat anti-rat IgG labeled with fluorophore Alexa 488, goat anti-rabbit IgG labeled with fluorescence Alexa 555 (Proteintech, USA, RGAR003), and goat anti-mouse IgG labeled with fluorescence Alexa 546 (Invitrogen, Waltham, MA, USA, A11003)). After three washes in PBS and subsequent staining with Hoechst, the sections were captured using a CCD camera mounted on a fluorescence microscope.

2.4 Results

Generation and evaluation of monoclonal antibodies against UBAP2Z and UBAP2W

Monoclonal antibodies against UBAP2Z and UBAP2W were generated to confirm the expression of each protein. Their specificity was examined by ICC and western blotting using recombinant protein expressed in COS7 cells (Fig. 9). Both assays demonstrated that each antibody specifically recognized its corresponding recombinant protein. Clone number #24-1 of UBAP2Z antibody, #2-6-7 of UBAP2W antibody for western blotting and #2-29-3 of UBAP2W antibody for IHC were chosen. In western blotting, the full-length recombinant proteins of UBAP2Z and UBAP2W were detected at approximately 180 kDa. These results indicated that the antibodies were suitable for subsequent analyses using embryonic gonads of the Japanese quail.

UBAP2Z and UBAP2W proteins are expressed in embryonic gonads

To confirm endogenous protein expression of UBAP2Z and UBAP2W, Western blotting was performed on extracts from embryonic gonads (Fig. 10A, B). UBAP2Z was detected at approximately 180 kDa in both sexes at HH27, HH31, HH34, and HH38, with strong signal observed at HH31 (Fig. 10A). By contrast, UBAP2W was most strongly detected

at approximately 180 kDa in females at HH27, with weaker expression at HH31, HH34, and HH38 (Fig. 10B). The molecular weights of both UBAP2Z and UBAP2W were higher than the predicted molecular weight (~120 kDa) based on the amino acid sequences. However, these were detected at the same molecular weights with full-length recombinant proteins of UBAP2Z and UBAP2W. These expression patterns were consistent with the mRNA expression profiles predicted from TPM values.

UBAP2Z and UBAP2W interact with distinct proteins in gonads

Because both UBAP2Z and UBAP2W contain the UBA domain, they may interact with ubiquitinated proteins in gonads. To explore potential protein–protein interactions, far-western blotting was performed using HH38 gonad extracts and recombinant UBAP2Z or UBAP2W (Fig. 10C). A single band was detected at ~50 kDa in both sexes using UBAP2Z recombinant protein, whereas a single band was detected at ~52 kDa specifically in females using UBAP2W recombinant protein. These results suggest that UBAP2Z and UBAP2W associate with different binding partners in gonads. Collectively, the findings imply that UBAP2Z functions similarly in both sexes, whereas UBAP2W may exert a female-specific role.

Protein localization of UBAP2Z and UBAP2W differed in embryonic gonads

To confirm the expression patterns of UBAP2Z and UBAP2W in embryonic gonads, IHC was performed using their respective monoclonal antibodies along with antibodies against cell marker proteins: DDX4 as a marker of germ cells and SOX9 as a marker for Sertoli cells.

In male gonads, IHC of UBAP2Z and DDX4 confirmed UBAP2Z expression in germ cells in the seminiferous tubules at HH27, HH31, HH34, and HH38 (Fig. 11A). All DDX4 signals in each HH stage overlapped with UBAP2Z signals. IHC of UBAP2Z and SOX9 showed no signal overlap; UBAP2Z was localized to the cytoplasm while SOX9 was confined to the nucleus (Fig. 11B). This spatial arrangement suggests that UBAP2Z is expressed surrounding the SOX9-positive nuclei in Sertoli cells of male gonads.

In female gonads, UBAP2Z was broadly expressed throughout gonads at HH27 and HH31, but its localization shifted to medullary somatic cells at HH34 and HH38 (Fig. 11C). All DDX4 signals in HH27 and HH31 overlapped with UBAP2Z signals. Notably, UBAP2Z was not expressed in germ cells at these late stages. By contrast, IHC of UBAP2W and DDX4 confirmed UBAP2W expression in germ cells across all developmental stages in female gonads (Fig. 11D). All DDX4 signals in each HH stage

overlapped with UBAP2W signals. Like UBAP2Z, UBAP2W was localized to the cytoplasm. Although UBAP2W was also observed in somatic cells at HH27, HH31, and HH34, its expression in medullary somatic cells disappeared at HH38.

These results suggest that UBAP2W may be involved not only in ovarian differentiation during early stages (HH27-HH34), but also in oogenesis at HH38. As gonadal differentiation progressed, the expression patterns of UBAP2Z and UBAP2W changed dynamically. In female gonads at HH38, UBAP2Z was expressed in medullary somatic cells, while UBAP2W was specifically expressed in cortical germ cells. Therefore, I concluded that UBAP2W performs a different role than UBAP2Z, particularly in germ cells.

2.5 Discussion

Although several studies have investigated W-linked genes other than *UBAP2* in chickens, detailed protein analysis between the Z and W alleles has not been performed[106-108]. Because mRNA and protein expression levels often do not correlate[109,110], direct protein analysis is essential. In my study, UBAP2Z and UBAP2W proteins were detected in gonads at HH27, HH31, HH34 and HH38, with UBAP2Z expressed in both sexes and UBAP2W restricted to females. This result correlates with the expression of their mRNA in the gonads. Numerous splicing variants and isoforms of both chicken UBAP2Z and UBAP2W are predicted[111,112]. I expected to detect UBAP2Z and UBAP2W with various molecular weights. However, UBAP2Z and UBAP2W were mainly detected at 180 kDa in both sexes. This suggests that the 180 kDa UBAP2Z and UBAP2W proteins primarily function in the gonads. The molecular weights of detected UBAP2Z and UBAP2W were larger than the predicted from amino acid sequences. Intrinsically disordered proteins are detected 1.2-1.8 times higher than the predicted from amino acid sequence[113]. This is mainly due to the compositional bias typical of intrinsically disordered proteins. As predicted in mammalian UBAP2[55], UBAP2Z and UBAP2W is likely to have the intrinsically disordered regions. As a result, UBAP2Z and UBAP2W may have been detected at higher than the predicted. In addition to the results of protein interaction

analysis revealing that UBAP2Z and UBAP2W bind to different target proteins, these results suggest that UBAP2W mediates the degradation of different substrate proteins during gonadal differentiation distinct from that of UBAP2Z, potentially due to subtle differences in their UBA domains.

I observed that UBAP2Z was expressed in gonadal somatic cells of both sexes from HH27 to HH38, whereas UBAP2W expression was restricted to female somatic cells and disappeared by HH38. The fact that UBAP2Z expression is restricted to the cytoplasm of Sertoli cells and germ cells may suggest that proper proteolysis in these specific cells is performed for cell differentiation rather than for homeostatic survival. In female gonads, forkhead box L2 (FOXL2) and cytochrome P450 family 19 subfamily A member 1 (CYP19A1) are expressed in the nuclei of medullary somatic cells[114]. The ubiquitin–proteasome pathway itself is tightly regulated by E1, E2, and E3 ligases, and a previous study reported that the E2 ligase *UBE2I* is critical for female sex differentiation in chicken[115]. It has been revealed that the HINTW on the W chromosome interacts with UBE2I, suggesting that genes on the W chromosome function in sex differentiation[116]. UBAP2W expressed in medullary somatic cells of female gonads at HH27, HH31 and H34 may be involved in FOXL2-aromatase pathway. However, the gonads of *DMRT1* single copy knockout ZZ individuals in chicken differentiate into ovary but they are

infertile[117]. This suggests that, although sex determination does not require genes on the W chromosome, the W chromosome may be involved in ovarian differentiation and/or oogenesis.

UBAP2Z and UBAP2W were expressed in female germ cells from HH27 to HH34. Interesting feature of UBAP2Z and UBAP2W is that UBAP2Z expression disappeared by HH34, UBAP2W continued to be expressed in germ cells at HH38. Female PGCs differentiate into oogonia at HH34 and initiate meiosis around HH40[118]. In the gonads at HH38, the proteins interacting with UBAP2Z and UBAP2W are different, which may be due to the differentiation of female germ cells into oogonia. Protein degradation via the ubiquitin–proteasome pathway plays a crucial role in oocyte development in mammals[119]. Wnt/β-catenin pathway is involved in meiotic germ cell development[120] and is known to be strictly controlled by the ubiquitin proteasome pathway. Furthermore, the regulation of meiosis is governed by pathways that are distinct from those involved in mitosis. These observations suggest that UBAP2W may be involved in the regulation of meiosis in females, potentially by modulating protein degradation through the ubiquitin–proteasome pathway.

2.6 Tables

Table 4 Primers used in this chapter

Primer name	Experiment	Sequence 5' to 3'
2550F	sex check	GTTACTGATTCGTCTAGGAGA
2718R		ATTGAAATGATCCAGTGCTTG
UBAP2_F_5'UTR	Cloning	TGTATATGATGACTTCGGTG
UBAP2Z_R_stop		TTAGTTTGTCCAGTATGCAG
UBAP2W_F_5'UTR	Cloning	GGCTGGCTTCTTTCG
UBAP2W_R_stop		TTAGTTAGTCCAGTATGGAG
InFusion_UBAP2_F	Recombinant protein	CGGTACCCGGGGATCTATGATGACTTCGGTGGGC
InFusion_UBAP2ZW_FLAG_R		CTTTGTAGTCGTTWGTCCAGTATGCAGAAGTG
InFusion_FLAG_F		CTGGACWAACGACTACAAAGACCATGACGG
InFusion_FLAG_R		CAGGTCGACTCTAGAGGATCCTACTTGTCATCGTCATCCTTG
InFusion_UBAP2W_F		CGGTACCCGGGGATCGGCTGGCTTCTTTCG
M13 Forward	Sequence	GTAAAACGACGGCCAGT
M13 Reverse		CAGGAAACAGCTATGAC
UBAP2_R1		CCACGTCCAAAYCCTCTC
UBAP2_R2		TAAGTCATCATAGCCTGCTC
UBAP2_R3		GTTGGTTCAGATGGAGCC
UBAP2_F2		CCTTTGAATACCTCTTTGCC
UBAP2_seqF		AAGAGACAGAAGGCAACTC
UBAP2_seqR		CCCATACTGGAATGCAC

2.7 Figures

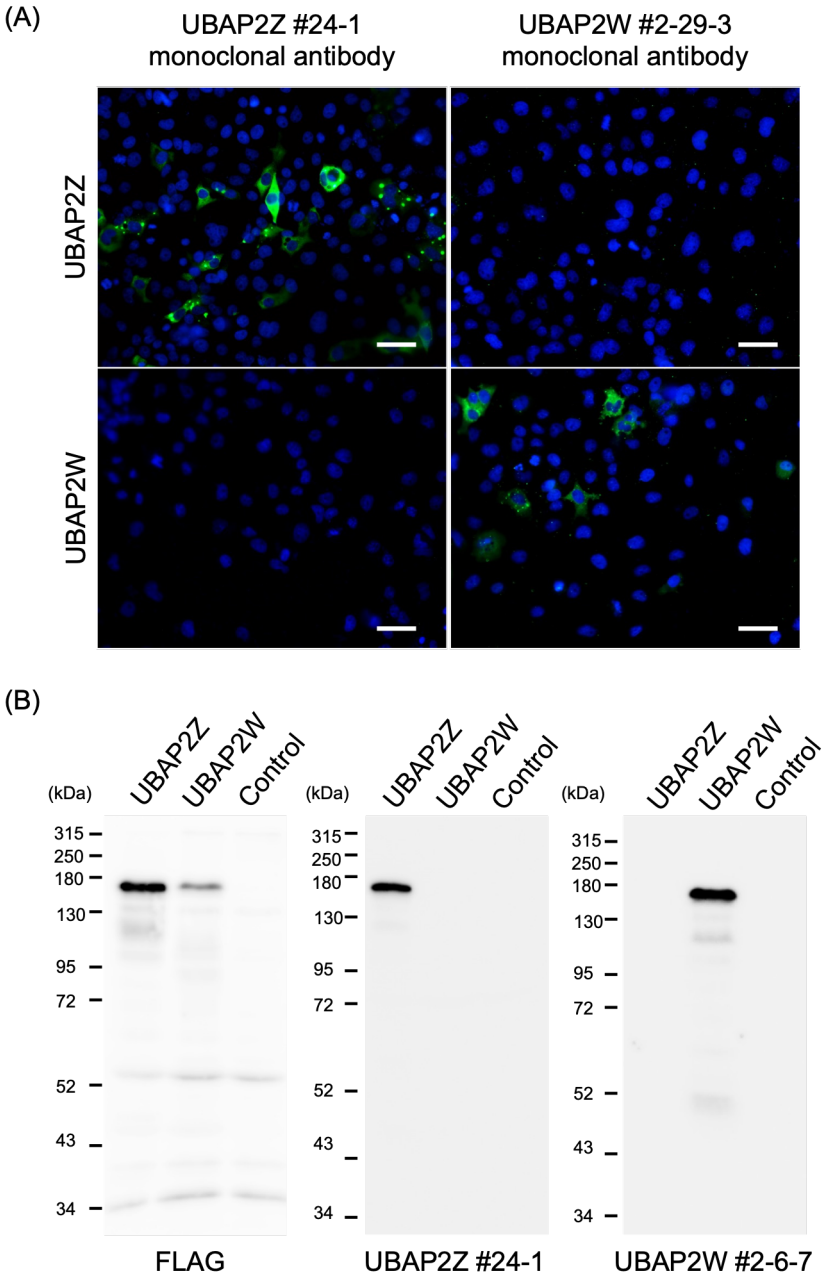

Fig. 9 Evaluation of generating monoclonal antibodies for western blotting and ICC

Validation of monoclonal antibodies using recombinant UBAP2Z and UBAP2W proteins expressed in COS7 cells. (A) ICC demonstrating the specificities of the monoclonal antibodies. Scale bars indicate 100 μm. (B) Western blotting demonstrating the specificities of the monoclonal antibodies.

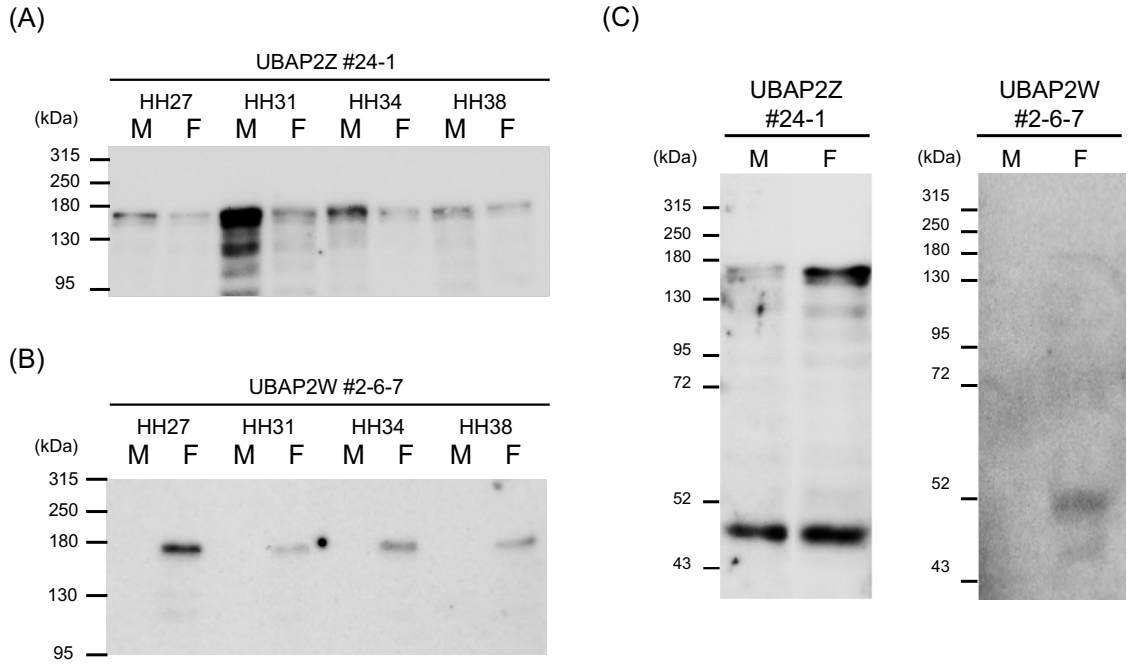

Fig. 10 Expression analysis of UBAP2Z and UBAP2W by western blotting

Western blotting analysis of embryonic gonads from male and female Japanese quail from HH27 to HH38. (A) Detection of UBAP2Z using a specific monoclonal antibody. (B) Detection of UBAP2W using a specific monoclonal antibody. (C) Protein–protein interaction analysis by far-western blotting using protein extracts of HH38 gonads of both sexes and recombinant UBAP2Z and UBAP2W proteins.

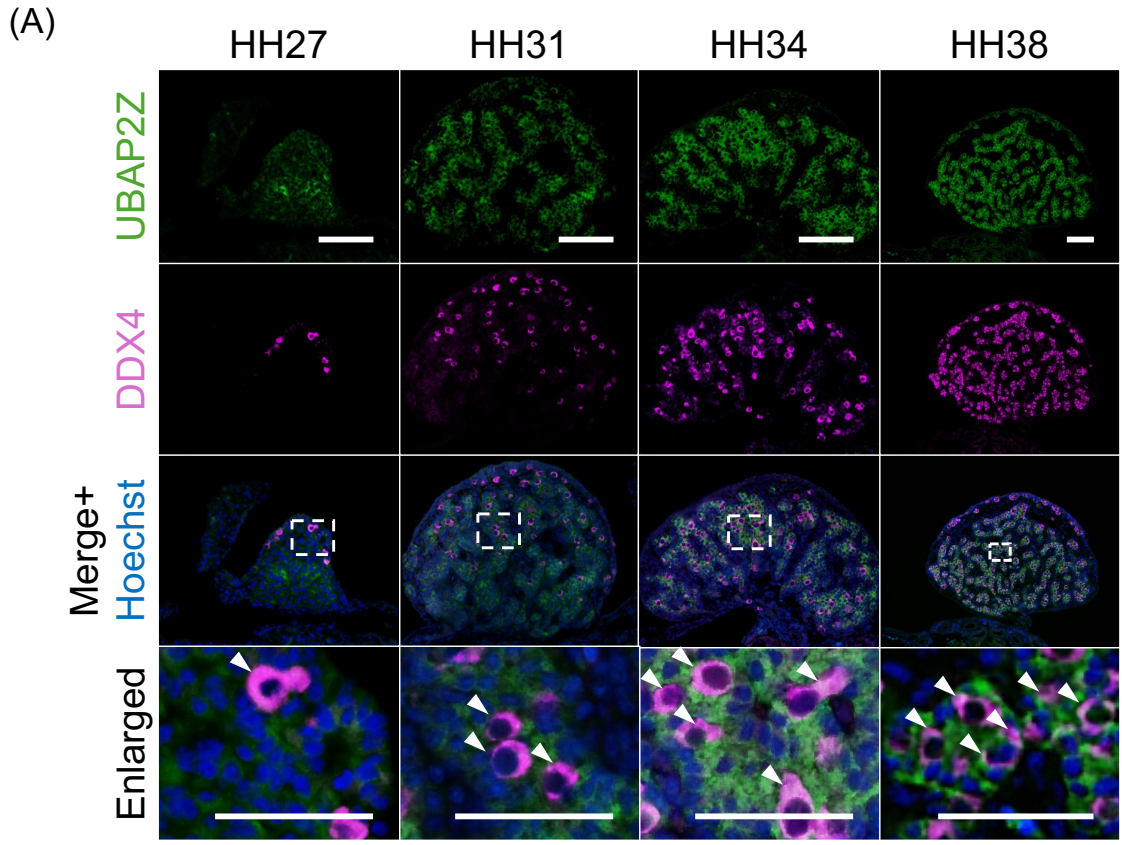

Fig. 11 IHC using gonads at HH27, HH31, HH34 and HH38

IHC of embryonic gonads from male and female Japanese quail from HH27 to HH38. (A) Double staining of UBAP2Z (green) and DDX4 (magenta), with nuclear counterstaining with Hoechst (blue) in male gonads. White arrowheads indicate overlap of UBAP2Z and DDX4. Scale bars indicate 100 μm. Scale bars of enlarged figures indicate 50 μm.

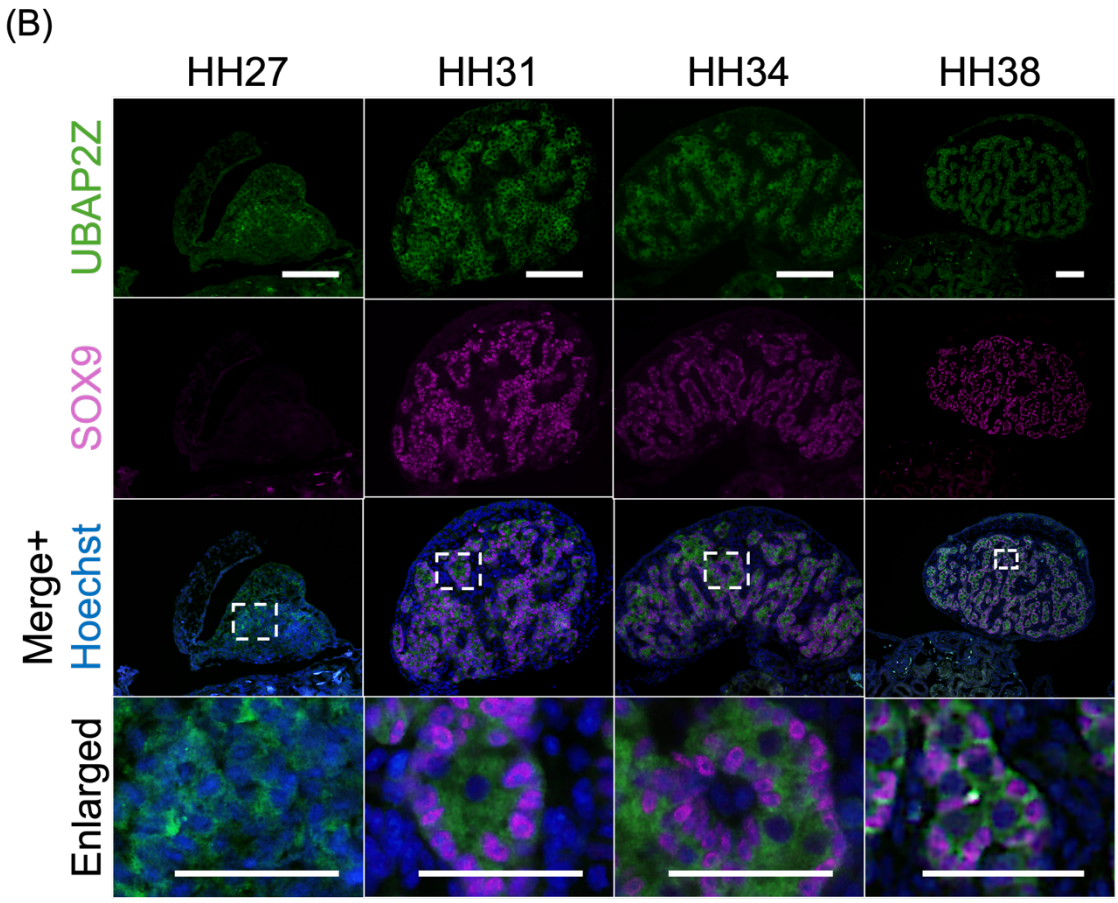

Fig. 11 IHC using gonads at HH27, HH31, HH34 and HH38 (Continued)

IHC of embryonic gonads from male and female Japanese quail from HH27 to HH38. (B) Double staining of UBAP2Z (green) and SOX9 (magenta) with Hoechst (blue) in male gonads. Scale bars indicate 100 μm. Scale bars of enlarged figures indicate 50 μm.

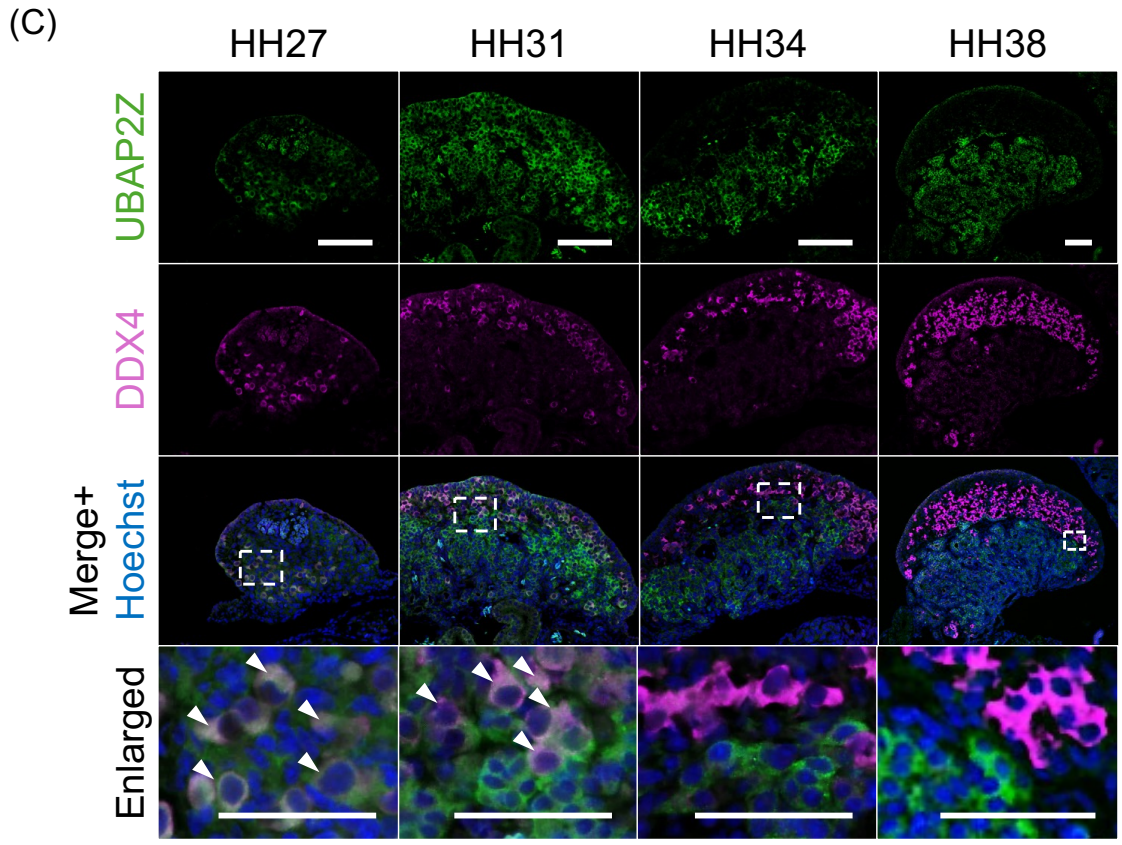

Fig. 11 IHC using gonads at HH27, HH31, HH34 and HH38 (Continued)

IHC of embryonic gonads from male and female Japanese quail from HH27 to HH38. (C) Double staining of UBAP2Z (green) and DDX4 (magenta) with Hoechst (blue) in female gonads. White arrowheads indicate overlap of UBAP2Z and DDX4. Scale bars indicate 100 μm. Scale bars of enlarged figures indicate 50 μm.

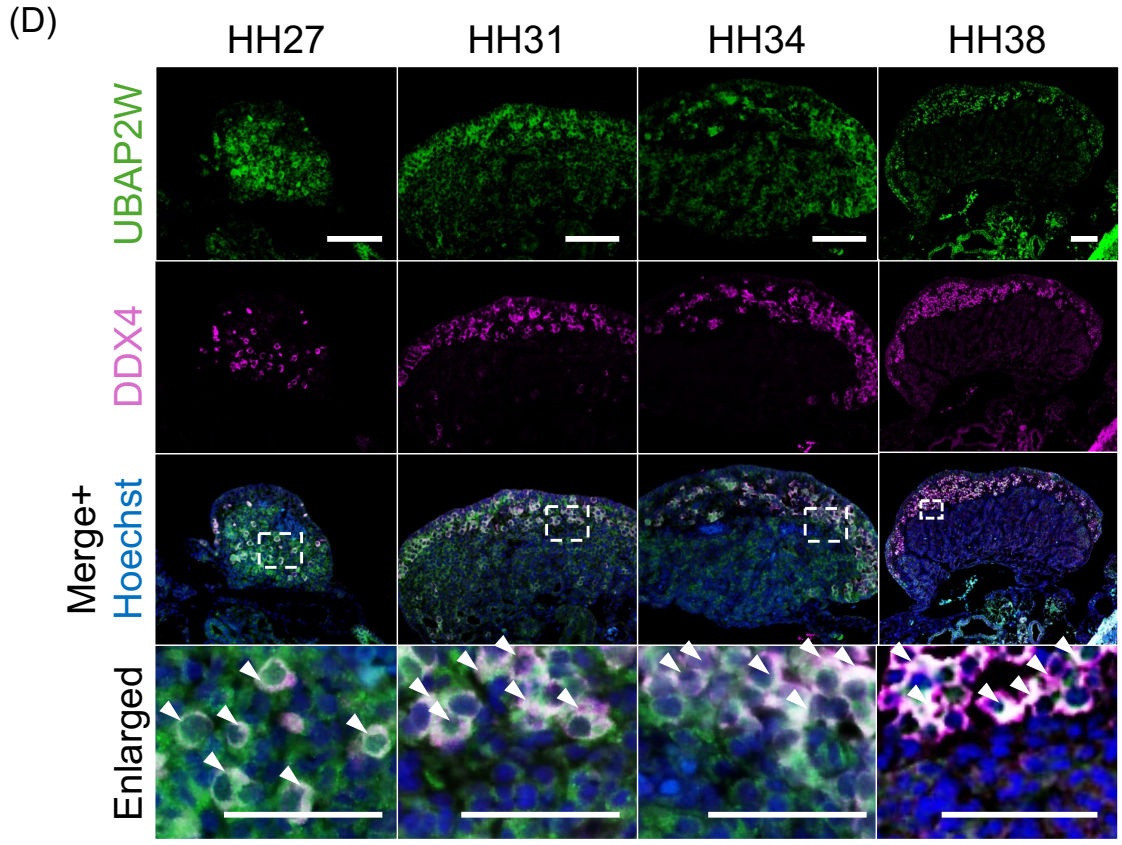

Fig. 11 IHC using gonads at HH27, HH31, HH34 and HH38 (Continued)

IHC of embryonic gonads from male and female Japanese quail from HH27 to HH38. (D) Double staining of UBAP2W (green) and DDX4 (magenta) with Hoechst (blue) in female gonads. White arrowheads indicate overlap of UBAP2W and DDX4. Scale bars indicate 100 μm. Scale bars of enlarged figures indicate 50 μm.

General discussion

In this study, I demonstrated that UBAP2W is functionally distinct from UBAP2Z, based on differences in mRNA expression patterns, amino acid sequences, protein interaction and protein localization. UBAP2W appears to have functionally diverged from UBAP2Z and is predicted to have acquired female-specific functions, potentially in sex determination and oogenesis, through its involvement in the ubiquitin-proteasome pathway. In addition, UBAP2W is expressed broadly throughout the body, suggesting that it may also retain an ancestral role in maintaining essential gene expression for survival.

UBAP2W is considered to be involved in sex determination, sex differentiation and oogenesis because it was expressed in both somatic cells and germ cells of the gonads from HH27 to HH38. Avian sex determination is thought to occur either through *DMRT1* dependent promotion of testis differentiation[121,122] or through W-linked genes that promote ovarian differentiation and/or suppress testis differentiation. Although *DMRT1* transcription levels are higher in males than in females, DMRT1 protein levels are equivalent between sexes during the early stages of gonadal development[123]. This discrepancy is thought to result from translational and post-translational regulation, after which DMRT1 protein levels decrease specifically in females. If UBAP2W has a role in sex determination, it may function in suppressing testicular differentiation through the

proteolytic degradation of testis differentiation genes via the ubiquitin-proteasome pathway. Previous studies across multiple taxa have shown that the ubiquitin-proteasome pathway contributes to sex determination.

In *C. elegans*, sex is determined by regulating the ubiquitin-proteasome degradation of sex determining transformer protein 1 (TRA-1), the final global sex-determining factor, via the cullin 2 (CUL-2) complex containing the FEM protein[124]. In mice, knockout of NEDD4 E3 ubiquitin protein ligase (NEDD4) impairs proliferation of gonadal progenitor cells by disrupting insulin and insulin growth factor 1 (IGF1) signaling through NEDD4 and its interacting protein growth factor receptor bound protein 10 (GRB10)[125], resulting in complete male-to-female gonadal sex reversal. Thus, it is possible that UBAP2W suppresses testicular differentiation by degrading factors involved in testicular differentiation in Japanese quail.

In mammalian sex differentiation, Wnt/β-catenin signaling promotes ovarian differentiation and inhibits testicular differentiation by repressing the expression of *Sox9*[126]. Although the regulatory mechanism is not fully resolved, the ubiquitin-proteasome pathway has been suggested to participate in this process. β-catenin directly binds the C-terminus of SOX9, resulting in β-catenin degradation through a ubiquitination/26S proteasome-dependent pathway[127]. In chickens, *UBE2I* is important

for female sexual differentiation[116], further supporting the idea that the ubiquitin-proteasome pathway contributes to ovarian differentiation. Because UBAP2W is broadly expressed in the female gonad at HH27 and HH31 in Japanese quail, it may promote ovarian differentiation by degrading proteins that favor testicular differentiation. To verify whether *UBAP2W* is involved in sex determination and sexual differentiation, it is necessary to identify proteins that interact with UBAP2W and analyze the effects of overexpressing *UBAP2W* in ZZ embryos on sex differentiation.

Evidence from gynandromorphic chickens[128] and chicken primordial germ cells (PGCs)[129–131] indicates that both somatic and germline sex differentiation rely on intrinsic chromosomal composition, a concept known as cell-autonomous sex identity (CASI). Moreover, the largely normal phenotype of *DMRT1* single-copy knockout chickens implies that the W chromosome plays a pivotal role in establishing this female identity[117]. Female PGCs are enriched in proteins associated with the ubiquitin-proteasome pathway compared with male PGCs[129], implying that this pathway is important for regulating female PGCs differentiation. *UBAP2W* is expressed in female PGCs from E2.5 to E6.5[132], suggesting that it may contribute to germ cell feminization by regulating the expression of female-specific proteins via the ubiquitin–proteasome pathway. To elucidate this

potential role, it will be necessary to examine changes in gene expression following *UBAP2W* is knocked down in cultured female PGCs.

At the same time, UBAP2W is expressed in a broad range of tissues, raising the possibility that it retains ancestral function similar to UBAP2Z. Both UBAP2Z and UBAP2W may play an essential role in survival. In mammals, the ubiquitously transcribed tetratricopeptide repeat containing, Y-linked (UTY), a homologue of the X-linked UTX, exhibits significantly lower histone demethylation activity than UTX[133,134]. Nevertheless, UTY interacts with chromatin-remodeling factors similar to those of UTX and can substitute for UTX's non-catalytic functions in UTX-deficient mice[133,135]. UBAP2W may compensate for the function of UBAP2Z and perform proteolysis that is important for embryonic development in birds. Although this study focused on the sex-specific gonads, comparative analyses of UBAP2Z and UBAP2W in tissues with minimal sex bias may clarify whether their functions are equivalent.

Overall, this study demonstrated clear differences in sequence, expression patterns, and protein localization between *UBAP2Z* and *UBAP2W*. This represents the first detailed protein level characterization of a W-linked gene in birds, revealing that the W-linked gene performs a female-specific function. These findings provide new insights into the evolution of avian sex chromosomes and the mechanisms underlying gonadal

development. Nevertheless, it still remains unclear whether *UBAP2W* directly contributes to sex differentiation or oogenesis. Gene knockout using genome editing represents the most promising approach for functional analysis. Currently, avian genome editing technology typically involves transferring CRISPR/Cas9-edited PGCs into recipient embryos[136]. However, this method has not yet been established for female PGCs, making it impossible to generate individuals with knockout of W-linked genes. Therefore, an alternative approach, other than the generation of chimeric individuals, is required to achieve W-linked gene knockouts. The genome editing technique has been established in the Japanese quail that enables gene knockout without generating chimeric individuals[137]. Using this technology, I could anticipate that it is able to generate *UBAP2W* knockout individuals. Additionally, identifying proteins that interact with UBAP2W will be essential for elucidating its molecular mechanisms. Further functional analyses are therefore necessary to clarify the role of *UBAP2W* in ovarian differentiation and oogenesis.

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Main publication

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Presentations

Oral Presentations

○**程田 大智**、水島 秀成、黒岩 麻里、ニホンウズラ(*Coturnix japonica*)における性染色体連鎖遺伝子 *UBAP2* の発現解析、染色体学会第 74 回年会、オンライン、2023 年 10 月

○**程田 大智**、水島 秀成、黒岩 麻里、ニホンウズラ(*Coturnix japonica*)における性染色体連鎖遺伝子 *UBAP2* の発現解析、日本動物学会北海道支部第 68 回大会、札幌、2024 年 3 月

Poster Presentations

○**程田 大智**、水島 秀成、奥野 未来、伊藤 武彦、黒岩 麻里、ニホンウズラ(*Coturnix japonica*)における W 染色体遺伝子の機能解析、染色体学会第 72 回年会、オンライン、2021 年 9 月

○**程田 大智**、水島 秀成、黒岩 麻里、ニホンウズラ(*Coturnix japonica*)における性染色体連鎖遺伝子 *UBAP2* の発現解析、日本分子生物学会第 46 回年会、神戸、2023 年 12 月

○**程田 大智**、水島 秀成、小林 朋絵、松山 誠、黒岩 麻里、ニホンウズラ生殖腺における *UBAP2Z* と *UBAP2W* の機能分化、日本分子生物学会第 48 回年会、横浜、2025 年 12 月