Sex Determination in Birds: 
*HINTs from the W Sex Chromosome?

C.A. Smith 

University of Melbourne, Department of Paediatrics and Murdoch Children’s Research Institute, Royal Children’s Hospital, Parkville, Victoria, Australia

**Key Words**

ASW · Chicken sex determination · HINTW · W sex chromosome · WPKCI

**Abstract**

Sex in birds is controlled genetically (ZZ male: ZW female), but the genetic mechanism remains unclear. While some evidence points to the involvement of Z sex chromosome dosage, other data favour a dominant female-determining gene carried on the W sex chromosome. An intriguing candidate gene located on the chicken W chromosome is HINTW, which encodes an aberrant form of a hydrolase enzyme. In chicken embryos, HINTW is strongly expressed in the gonads and other tissues of ZW (female) embryos. In vitro biochemical data show that HINTW can interfere with the action of a Z-linked orthologue, HINTZ, which is a bona fide hydrolase enzyme. HINTW is conserved among carinate (flying) birds, and recent molecular analysis indicates that it has undergone positive selection over evolution. However, a differentiated HINTW gene appears to be absent in the flightless ratites. This review examines the evidence for and against a role for HINTW in avian sex determination.

Sex is determined in vertebrates by either genetic or environmental cues. Many reptiles exhibit temperature-dependent sex determination, whereby the temperature of egg incubation controls sex [reviewed in Pieau and Dorizzi, 2004]. In contrast, all mammals and birds have so-called genotypic sex determination (GSD), in which sex is controlled by the inheritance of sex chromosomes. In therian mammals, the Y-linked SRY gene directs testis formation in XY embryos, while ovary formation proceeds in XX embryos. The sex chromosomes of birds are widely thought to have evolved from a different autosomal pair to those of mammals, and as such are designated ‘Z’ and ‘W’. The platypus, a primitive monotreme mammal, has a bizarre series of five sex chromosome pairs, with both therian X and avian Z-related elements, suggesting an association between the ZW and XY systems in the ancestors of birds and mammals [Grutzner et al., 2004; Ezaz et al., 2006]. In a pattern that is opposite to that of mammals, female is the heterogametic sex in birds (ZW) while the male is homogametic (ZZ). Despite a number of years of research on avian sex determination, the genetic mechanism responsible for directing male versus female development is still unknown. The Z dosage hypothesis postulates that the dosage of a Z-linked gene underlies sex determination in birds, with two doses being required for male development. Meanwhile, the dominant W hypothesis postulates that an ovary determinant is carried on the female (W) sex chromosome. These two hypotheses are not necessarily mutually exclusive. In this review, the latest evidence in support of the dominant W hypothesis is discussed, with particular reference to the candidate W-linked gene, HINTW.
Does the Avian W Sex Chromosome Have a Role in Sex Determination?

The potential role of the avian W chromosome in sex determination could be readily established by the sexual phenotype of sex chromosome aneuploids (2A:ZZW or 2A:ZO). However, such aneuploidy has not been definitively documented in birds [reviewed in Smith and Sinclair, 2004], and indeed it may be embryo lethal [Graves, 2003]. In an evolutionary context, sex chromosomes are thought to arise when one chromosome acquires a sex-determining function, and other genes enhancing fitness for that sex accumulate around it, favouring suppression of recombination with its homologue [Ohno, 1967; Charlesworth, 1991]. Genetic isolation then results in the accumulation of repetitive elements and ‘degeneration’ of the non-recombining differentiated sex chromosome. In general terms, this can explain the evolution of the Y versus the X chromosome in therian mammals. If this has also occurred in birds, then the W chromosome has become differentiated from the Z because it carries an ovary (female) determining gene. An alternative scenario, however, is that the chromosome pair which gave rise to the avian Z and W chromosomes shared a gene that was lost from one homologue, leading to a sex-determining system based on gene dosage [Marshall Graves and Shetty, 2001]. The Z-linked candidate male gene, DMRT1, for example, is present in two copies in males (ZZ) but as one copy in females (ZW), in all birds that have been examined. In the chicken, DMRT1 is expressed in embryonic gonads, as expected of a sex determinant [Raymond et al., 1999; Smith et al., 1999; Shan et al., 2000; Zhao et al., 2007]. However, while it is always more highly expressed in males (ZZ), gonadal DMRT1 expression from embryonic day 3 precedes the onset of gonadal sex differentiation in the chicken embryo by at least 3 days (differentiation begins at the histological level from day 6 [Smith et al., 2007]). This would indicate that either DMRT1 does not play a role in initiating gonadal sex differentiation, or that its early expression sets in motion a series of molecular events that are not apparent morphologically until some time later (from embryonic day 6).

Could the Z chromosome dosage and dominant W hypotheses be both correct? It is difficult to see how both mechanisms could have evolved, because the advent of one mechanism would be strongly selected for and driven to stability, with no selective pressure for the alternative. It therefore seems more likely that one or other of these mechanisms has prevailed. In therian mammals, current evidence indicates that the Y chromosome is differenti-
Table 1. Genes of known or predicted function on the chicken (Gallus gallus) W sex chromosome

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Encoded protein</th>
<th>Function</th>
<th>Accession number</th>
<th>Z homologue</th>
</tr>
</thead>
<tbody>
<tr>
<td>HINTW</td>
<td>Histidine triad nucleotide binding protein, W-linked</td>
<td>Hydrolysis of AMP; Potential sex determinant</td>
<td>NM_204688</td>
<td>Yes</td>
</tr>
<tr>
<td>FET1</td>
<td>Female expressed transcript 1</td>
<td>Unknown</td>
<td>NM_204544</td>
<td>No</td>
</tr>
<tr>
<td>2d-2D9</td>
<td>Not known</td>
<td>Unknown</td>
<td>AB188526</td>
<td>Unknown</td>
</tr>
<tr>
<td>2d-2F9</td>
<td>AAA ATPase</td>
<td>ATPase</td>
<td>AB1888527</td>
<td>Yes</td>
</tr>
<tr>
<td>SPIN-W</td>
<td>Spindlin</td>
<td>Structural role in mitosis</td>
<td>NM_204191</td>
<td>Yes</td>
</tr>
<tr>
<td>ATP5A1-W</td>
<td>ATP synthase subunit alpha, gene exon 5</td>
<td>ATP synthesis coupled proton transport</td>
<td>XM_429118</td>
<td>Yes</td>
</tr>
<tr>
<td>CHD1-W</td>
<td>Predicted: chromodomain helicase DNA binding protein 1</td>
<td>DNA/chromatin binding</td>
<td>XM_424694</td>
<td>Yes</td>
</tr>
<tr>
<td>AKAP8L</td>
<td>A kinase (PRKA) anchor protein 8-like</td>
<td>A protein of the chromatin and nuclear matrix; DNA-binding; zinc ion binding</td>
<td>NM_001031366</td>
<td>No, Autosomal?</td>
</tr>
<tr>
<td>LOC769313b</td>
<td>Predicted: Pro-Pol-dUTPase polypeptide; RNaseH; dUTPase; integrase; protease; reverse transcriptase</td>
<td>DNA binding; Ribonuclease H activity</td>
<td>XM_001232569</td>
<td>Yes</td>
</tr>
<tr>
<td>LOC769000b</td>
<td>Predicted: similar to MADH2 protein</td>
<td>SMAD family of TGFβ; Signal transduction, DNA transcriptional regulation</td>
<td>XM_001232180</td>
<td>Yes</td>
</tr>
<tr>
<td>LOC431005b</td>
<td>Predicted: similar to Potassium channel modulatory factor 1</td>
<td>Cellular physiology</td>
<td>XM_428553</td>
<td>Yes</td>
</tr>
<tr>
<td>LOC426031b</td>
<td>Predicted: similar to delangin isoform A; Nipped-B-like</td>
<td>Regulation of chromosome activity</td>
<td>XM_423708</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Another 20–30 genes of unknown function have been tentatively assigned to the chicken W chromosome.

* Also called ASW (Avian Sex-specific, W-linked) and WPKCI (W-linked, Protein Kinase C Inhibitor).

Candidate Sex Genes on the W Chromosome

Cross-species chromosome painting and gene mapping have shown that the Z sex chromosome is highly conserved among birds (including the flightless ratites, which have homomorphic sex chromosomes), while the smaller heterochromatic W is less well conserved [Ogawa et al., 1998; Shetty et al., 1999; reviewed in Stiglec et al., 2007a]. It seems likely that the avian W chromosome has undergone degenerative changes independently among the various orders of birds. Like the mammalian Y chromosome, the W is largely heterochromatic and has a number of repetitive elements [Takagi and Sasaki, 1974; Tone et al., 1982]. In the chicken, Gallus gallus, up to 70% of the W chromosome is composed of repetitive DNA [Itoh and Mizuno, 2002]. The genome of this species is the first among birds to have been fully sequenced [International Chicken Genome Sequencing Consortium, 2004]. A number of chromosomal regions and genes originally assigned to the chicken W chromosome on the basis of associated repetitive DNA have recently been reassigned to the Z sex chromosome by metaphase FISH [Stiglec et al., 2007b]. This leaves few bona fide genes on the chicken W. Five known genes have been physically mapped to the chicken W chromosome, with another thirty or so putative W-linked sequences encoding proteins of predicted or unknown function, according to the most recent build of the chicken genome on NCBI [http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9031; Schmid et al., 2000, 2005; table 1]. Given the recent reassignment of many putative W genes to the Z, this small list may shorten further when more extensive mapping is carried out. Almost all of the known W-linked genes have homologues on the Z and they encode proteins with structural or ‘house-keeping’ type roles in the cell (table 1), suggesting that they do not play a sex-specific role. One predicted gene, the anchor protein encoded by AKAP8L, does not appear to have a Z homologue, but autosomal homologues exist. However, this may reflect a mis-assignment or gaps in the Z sequence.

Two genes do not appear to have Z homologues: FET1 and 2d-2D9 [Reed and Sinclair, 2002; Yamada et al., 2004].
**FET1** (female expressed transcript #1) is expressed in the female urogenital system of chicken embryos at the time of sexual differentiation. Recent sequence analysis of this gene suggests that it encodes an avian retroviral element, which may be un-related to sex determination. The urogenital system appears to be particularly favourable for the expression of endogenous retroviral sequences [C. Smith, pers. obs.]. Furthermore, **FET1** is asymmetrically expressed, only in the left gonad of female embryos, while early male markers (e.g., aromatase) are expressed in both left and right gonads. **FET1** expression is also unaffected during female-to-male sex reversal induced by inhibition of oestrogen synthesis [Zheng and Yang, 2007]. Together, these observations suggest that **FET1** may be un-related to sex determination in the chicken embryo. Yamada et al. [2004] isolated 2 novel W-linked cDNAs from embryonic chicken gonads, which they termed 2d-2D9 and 2d-2F9. Recent data indicate that the latter encodes an ATPase, it has a Z-linked homologue and it is ubiquitously expressed in embryos [Koyama et al., 2007], making a sex-specific role unlikely. However, 2d-2D9 has no significant homology to other sequences on the chicken database, including no obvious Z homologue, making it a potential candidate female gene. However, this gene is expressed very early in female embryos, from at least day 2, which precludes formation of the gonadal primordium.

**HINTW: The Best Candidate Female-Determining Gene**

One other W-linked gene in birds remains the strongest candidate for a female determinant (table 1). This gene was isolated independently by two groups, based on differential (male versus female) cDNA screening of embryonic chicken gonads. The novel gene was originally called ASW (Avian Sex-specific, W-linked) [O’Neill et al., 2000] or WPKCI (W-linked, Protein Kinase C Inhibitor) [Hori et al., 2000]. The first name ascribed no particular function, while the second was based on weak homology to a protein kinase inhibitor. More recently, the gene has been re-named **HINTW**, Histidine triad Nucleotide binding protein, W-linked. This name is a more accurate description of the gene and has been adopted herein. **HINT** genes encode a family of nucleotide hydrolase enzymes that have a characteristic histidine triad (HIT) motif (His-x-His-x-His-xx, where x represents a hydrophobic amino acid). HINT enzymes function to hydrolyse the molecule adenosine-5’ monophosphoramidate (AMP-NH$_2$) or AMP-linked to lysine [Brenner, 2002; Krakowiak et al., 2004]. The HINT proteins act as homodimers to interface with their nucleotide substrates. However, **HINTW** is very unusual because it specifically lacks the key histidine triad motif that confers enzyme function [Hori et al., 2000; O’Neill et al., 2000] (fig. 1). Hori and colleagues [2000] showed that a bona fide **HINT** gene resides on the Z (now called **HINTZ**). **HINTZ** does contain a HIT motif and is predicted to encode a typical HINT protein (83% identical to rabbit HINT) [Hori et al., 2000]. **HINTZ** must represent the homologue of the altered W form. Outside the HIT motif, and a leucine/arginine rich region in **HINTW**, the two predicted proteins are well conserved (fig. 1). **HINT** genes are present in a wide variety of organisms, from prokaryotes through to humans, although **HINTW** is unique to birds.

Being W-linked, **HINTW** is restricted to females. The gene is widely expressed in female chicken embryos, with high expression in the gonads, and in the central nervous system and myotomes. In the whole embryo and in the gonads, **HINTW** expression is higher in earlier stage embryos leading up to gonadal sex differentiation [Hori et al., 2000; O’Neill et al., 2000]. **HINTW** has a similar expression profile. Being Z-linked, it is expressed in the gonads of both sexes, but at higher levels in males. Hori et al. [2000] found that **HINTW** is reiterated at least 40 times on the chicken W sex chromosome, although some copies of the gene differ slightly in sequence from **HINTW**. Southern blotting has revealed that **HINTW** is conserved and reiterated among carinate (flying) birds (table 2). However, in the emu and ostrich (basal, flightless birds), Southern blots of genomic DNA probed with chicken **HINTW** produce a single band in both sexes. This suggests that **HINTW** is not differentiated in rattites (not present) or that it is very similar to **HINTZ**. Ratite **HINT** genes have not yet been sequenced to clarify this point.
<table>
<thead>
<tr>
<th>Order</th>
<th>Species</th>
<th>Female specific band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galliformes</td>
<td>Domestic chicken, green pheasant, Japanese quail</td>
<td>+</td>
</tr>
<tr>
<td>Anseriformes</td>
<td>Domestic duck</td>
<td>+</td>
</tr>
<tr>
<td>Coraciformes</td>
<td>Kookaburra</td>
<td>+</td>
</tr>
<tr>
<td>Columbiformes</td>
<td>Rock dove, New Zealand pigeon</td>
<td>+</td>
</tr>
<tr>
<td>Strigiformes</td>
<td>Snowy owl</td>
<td>+</td>
</tr>
<tr>
<td>Gruidae</td>
<td>Japanese crane, moorhen, swamp hen</td>
<td>+</td>
</tr>
<tr>
<td>Sagittariiformes</td>
<td>Secretary bird</td>
<td>+</td>
</tr>
<tr>
<td>Charadriiformes</td>
<td>Black stilt, brown skua</td>
<td>+</td>
</tr>
<tr>
<td>Psitaciformes</td>
<td>Kakapo, scarlet macaw, crimson rosella</td>
<td>+</td>
</tr>
<tr>
<td>Passeriformes</td>
<td>Fairy wren, common finch, Java sparrow</td>
<td>+</td>
</tr>
<tr>
<td>Sphenisciformes</td>
<td>Adelie penguin, king penguin</td>
<td>+</td>
</tr>
<tr>
<td>Procellariiformes</td>
<td>Grey-faced petrel, streaked shearwater</td>
<td>+</td>
</tr>
<tr>
<td>Struthioniformes</td>
<td>Emu and ostrich</td>
<td>–</td>
</tr>
</tbody>
</table>

Biochemical Evidence of a Dominant Negative Role for HINTW in Avian Sex Determination

It has been postulated that HINTW may function as a dominant negative in avian sex determination, by forming heterodimers with HINTZ, thereby interfering with its function and inducing female development [Hori et al., 2000; O’Neill et al., 2000]. Pace and Brenner [2003] noted that 15 of the 16 normally conserved amino acid residues required for adenosine monophosphate (AMP) recognition by HINT enzymes are mutated in the predicted HINTW protein. Using molecular modelling, they inferred that HINTW titrates out HINTZ function through heterodimer formation, probably through the amino acid Gln27, which is substituted for Trp123 in HINT (Trp123 is the only one of the 16 amino acids required for substrate recognition that actually spans the dimer interface, and is therefore vulnerable to HINTW interference). This would be sufficient to block Histidine triad function in HINTZ, leading to a loss of AMP binding. Biochemical studies using site-directed mutagenesis have demonstrated that a Trp to Gln mutation at position 123 in HINT does indeed dramatically reduce its substrate specificity by 2,700-fold [Parks et al., 2004]!

Most recently, Moriyama and colleagues [2006] expressed epitope tagged HINTZ and HINTW proteins in E. coli, and demonstrated heterodimer formation in vitro using GST pull-down assays. They also showed that expressed HINTZ homodimers could hydrolyse adenosine 5’-monophosphoramidate, a function inhibited by the formation of a heterodimer with over-expressed HINTW. There is now good evidence, then, that HINTW can block HINTZ function, at least in vitro, conceivably inducing a downstream sex-specific pathway. But what are the targets of these enzymes in vivo? The in vitro substrate used in biochemical assays, AMP-NH₂, is a naturally occurring molecule in the cell. However, AMP-linked to lysine residues in key proteins could also be substrates. For example, the yeast homologue, Hnt1, acts as a positive regulator of Kin28, the kinase component of the general transcription factor TFIIH [Bieganowski et al., 2002]. It is suggested that Kin28 is post-translationally adenylated, and therefore represents a protein substrate for the AMP hydrolysis function of Hint [Brenner, 2002]. Intriguingly, the human HINT protein, HINT1, has recently been shown to regulate the function of the microphthalmia transcription factor (MITF). Mutations of Mitf in mice cause microphthalmia, deafness and hypopigmentation. In humans, MITF mutations cause Waardenburg syndrome type II, with similar eye and pigmentation anomalies. In mast cells, human HINT binds to MITF, blocking its function. However, HINT binding is dissociated by an exotic endogenous nucleotide, ApApApA (where A = adenosine). This dissociation allows MITF to activate downstream target genes [Lee et al., 2004]. Altogether, these data indicate that HINT proteins may be potent regulators of gene transcription. Therefore, the HINTZ/HINTW interaction in birds could potentially regulate the expression of downstream genes. However, the mouse homologue, Hint1, has been knocked out and is dispensable for development [Korsisaari et al., 2003]. It is possible that functional redundancy may apply, as there are several Hint-related genes in the genome.
Genetic Evidence in Support of **HINTW**

In the chicken, the **HINTW** gene is located on the non-heterochromatic tip of the W chromosome. This part of the W is non-recombining with the Z. This suggests that it has been specifically protected at this site. By studying the ratios of synonymous and non-synonymous amino acid substitutions, Ceplitis and Ellegren [2004] provide evidence that **HINTW** has undergone adaptive molecular evolution, implying strong selective pressure to isolate a female-specific function. Furthermore, amplification of **HINTW** could reinforce its potential role in female development. Note that a similar process has occurred in mammals; several spermatogenesis genes are repeated along the male-specific Y chromosome. In addition, it has been argued that the amplification of **HINTW** over 40 times on the W could provide a means of protecting the function of the gene against deleterious mutations that otherwise accumulate on the non-recombining region on the W sex chromosome [Backström et al., 2005]. This again points to a functional role for **HINTW** in development.

The Case against **HINTW**

There are some lines of evidence that do not support a role for **HINTW** in regulating avian sex determination. Firstly, the expression profile of **HINTW** in chicken embryos is not entirely consistent with a role in female gonadal sex differentiation. **HINTW** expression has been detected as early as day 2 in chicken embryos [Yamada et al., 2004], which is prior to formation of the gonads (day 3), and well before sexual differentiation (from day 6). It is difficult to envisage a role for **HINTW** in triggering ovary formation from this early time point, unless molecular events pre-date morphological events by a number of days. Recent data also indicate that **HINTW** expression does not decline in ZW embryos that undergo female-to-male sex reversal induced by blocking aromatase gene expression, as might be expected if the gene has a female-specific role [Zheng and Yang, 2007]. Furthermore, **HINTW** mRNA is expressed throughout the embryo in extragonadal sites, such as the central nervous system and myotomes [Scholz et al., 2006]. Therefore, if **HINTW** is translated into a functional protein, it must have other (female specific?) roles outside the gonads. It is possible that the protein has a feminising effect not only in the gonads, but in other sexually dimorphic tissues, such as the brain [Scholz et al., 2006; Smith et al., 2007].

Although it has been artificially over-expressed in bacteria, an endogenous **HINTW** protein has not yet been demonstrated. It is possible that the **HINTW** transcripts seen in gonads and other tissues are not translated. Perhaps the most significant evidence against a role for **HINTW** in avian sex determination is its apparent absence in ratites (emu and ostrich). In the context of vertebrate evolution, the ratites and carinates separated relatively recently (around 80 million years ago), and the Z sex chromosomes are highly conserved in these two bird groups. It therefore seems highly likely that the sex determinant in ratites is the same as that in carinates. Note that eutherian mammals and metatherians (marsupials) diverged at a much earlier time – around 180 mya – yet they still share the rapidly evolving **SRY** gene. Although Southern blots of DNA from emu and ostrich show a single band in both sexes, it will be of interest to sequence the **HINT** gene in these birds. The assumption at present is that **HINTW** is absent in ratites, and, if so, this makes it unlikely that the gene plays a role in avian sex determination.

The Verdict

While there is good in vitro biochemical data that artificially produced **HINTW** protein can interfere with **HINTZ** function, a role for the former in avian sex determination in vivo has not yet been proven. It has therefore not been established ‘beyond reasonable doubt’ that **HINTW** is the avian sex determinant. **HINTW** remains the best candidate under the dominant W hypothesis, but proof will ultimately come from the production of sex-reversed transgenic birds over-expressing the gene, and from knockdown studies in ovo.

References


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