Int. J. Dev. Biol. 53: 59-67 (2009)
doi: 10.1387/ijdb.082742cs

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Genetic evidence against a role for *W-linked histidine triad nucleotide binding protein (HINTW)* in avian sex determination

CRAIG A. SMITH*, KELLY N. ROESZLER and ANDREW H. SINCLAIR

Murdoch Childrens Research Institute and the Department of Paediatrics, The University of Melbourne, Parkville, Victoria, Australia

ABSTRACT Birds have a ZZ/ZW sex chromosome system, but the mechanism of sex determination remains unknown. The heterogametic sex is female (ZW) and one hypothesis holds that the W chromosome carries a dominant-acting ovary-determining gene. The strongest candidate ovary-determinant on the W chromosome is *HINTW*, which encodes an aberrant nucleotide hydrolase enzyme. *HINTW* is conserved amongst all carinate (flying) birds and it is strongly expressed in the gonads and other tissues of female chicken embryos. This and other lines of circumstantial evidence support the proposal that *HINTW* is the female-determining gene in birds. However, *in vivo* gain-of-function or loss-of-function studies have not hitherto been reported to test this hypothesis. We tested the potential role of *HINTW* by mis-expressing it in genetically male (ZZ) embryos, using the RCASBP avian retroviral vector. Strong, widespread expression was delivered throughout the embryo, including the urogenital system, as assessed by whole mount *in situ* hybridisation. This expression pattern mimicked that seen in normal ZW females, in which *HINTW* is widely expressed. Strong mis-expression was observed throughout the gonads of genetic male (ZZ) embryos. However, despite strong *HINTW* expression, ZZ gonads developed normally as bilateral testes. In tissue sections of ZZ urogenital systems transgenic for *HINTW*, normal testicular histology was observed. Female (ZW) gonads over-expressing *HINTW* also developed normally, with normal ovarian structure and left/right asymmetry. These results provide genetic evidence against a dominant role for *HINTW* in avian sex determination.

KEY WORDS: *HINTW, sex determination, bird, testis, ovary*

Introduction

Sex determination in higher vertebrates is controlled genetically by the inheritance of sex chromosomes. A gene or genes carried on the sex chromosomes initiate differentiation of the embryonic gonads into either testes or ovaries. While it was known for some time that mammals have an XX female/XY male sex chromosome system, it was not until 1990 that the master testis-determining gene, *SRY,* was discovered on the Y chromosome (Berta *et al.,* 1990; Koopman *et al.,* 1991; Sinclair *et al.,* 1990). In the absence of *SRY*, ovarian development occurs, as in XX embryos. A number of other genes downstream of *SRY* have since been identified in mammals (Wilhelm *et al.,* 2007), and some genes required for ovary development have also been discovered (Ottolenghi *et al.,* 2007; Parma *et al.,* 2006). In contrast, our understanding of sex determination and gonadal development in other vertebrates is less well understood. Sex is determined genetically in birds, as in mammals, but by a different pair of sex chromosomes, the Z and W. The female (ZW) is the heterogametic sex and the male (ZZ) is homogametic. However, the mechanism of sex determination in birds remains unknown. Two hypotheses have been proposed: female development is controlled by a key ovary-determining gene carried on the W sex chromosome, or gonadal development depends upon Z sex chromosome dosage (Ellegren, 2000; Smith *et al.,* 2007). Sex chromosome aneuploidy (in particular, 2A:ZO or 2A:ZZW) has not been definitively documented in birds, and indeed may be embryo lethal (Graves, 2003; Smith and Sinclair, 2004). A potential

Abbreviations used in this paper: HINTW, Histidine triad nucleotide binding protein,-W linked; LTR, long terminal repeat; RCASBP.B, replication-competent avian sarcoma-leukosis virus, long terminal repeat, with a splice acceptor, Bryan Polymerase, subtype B.

***Address correspondence to:** Craig Smith. Murdoch Childrens Research Institute and the Department of Paediatrics, The University of Melbourne, Parkville, Victoria, 3052, Australia. Fax: (+613)-8341-6429. e-mail: craig.smith@mcri.edu.au

Accepted: 6th August 2008. *Published online:* 18 November 2008. *Edited by:* Makoto Asashima.

ISSN: Online 1696-3547, Print 0214-6282

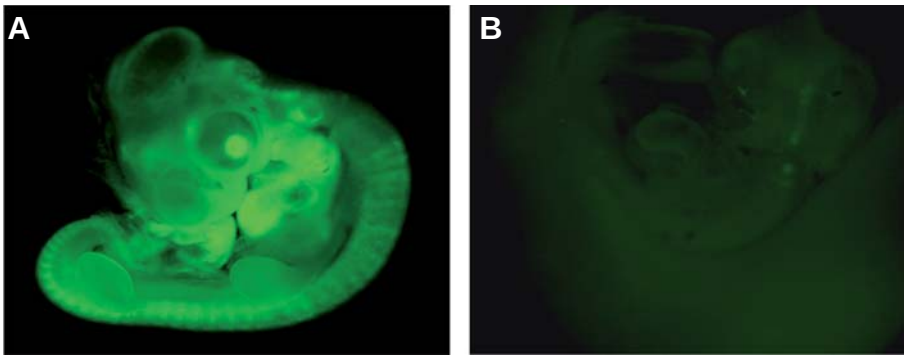


Fig. 1 (Above). Positive controls for RCASBP retroviral infection; global expression of eGFP in the chicken embryo. (A) *Day 4 embryo infected at day 0 with RCASBP.B.eGFP, showing ubiquitous eGFP expression.* **(B)** *No eGFP expression is detectable in uninfected negative control embryo.*

2A:ZZW female reed warbler has been described, supporting the dominant W hypothesis, although the bird was assessed by Z-linked microsatellites and karyotyping was not carried out (Arit *et al.,* 2004).

Under the Z dosage hypothesis, two copies of a Z-linked gene would result in testis development and male sex determination, while one copy would lead to ovarian development and female sex determination. The most favoured candidate gene under this hypothesis is *DMRT1*, which is Z-linked and male up-regulated in the embryonic chicken gonad (Nanda *et al.,* 2000; Raymond *et al.,* 1999; Shan *et al.,* 2000; Smith *et al.,* 2003). *DMRT1* encodes a transcription factor, characterised by a conserved DNA-binding motif, the DM domain (Murphy *et al.,* 2007). Homologues of *DMRT1* are involved in testicular development in other vertebrates (Kim *et al.,* 2007; Matsuda *et al.,* 2002; Matsuda *et al.,* 2007; Raymond *et al.,* 2000), while a female DM domain gene, *DM-W*, has recently been shown to participate in *Xenopus laevis* ovarian development (Yoshimoto *et al.,* 2008). However, it is now known that dosage compensation of Z-linked genes is weak in birds (Ellegren *et al.,* 2007; Itoh *et al.,* 2007; Melamed and Arnold, 2007), so that any Z-linked gene expressed in the embryonic gonads could theoretically regulate avian sex determination under the Z dosage hypothesis. The avian Z sex chromosome carries a large number of genes (Stiglec *et al.,* 2007), presenting a long list of potential candidates.

The alternative view of avian sex determination is that the W chromosome carries a dominant acting female determinant. Studies on triploid and chimeric chickens point to an ovary-determining gene on the W, but potentially counteracted by two Z chromosomes. For example, a line of triploid chicken with a 3A:ZZW genotype develop as intersexes, with a right testis and left ovotestis, but the ovarian component later regresses (Lin *et al.,* 1995; Thorne, 1997; Thorne and Sheldon, 1991). The phenotype of these birds implies that the W carries an ovary-determining gene that can be overcome by two copies of the Z sex chromosome. A diploid-triploid chimeric chicken with ZZ/ZZW sex chromosomes and a small proportion of triploid cells developed as a male, but with a left ovotestis and right testis, indicating that even a small number of W-bearing cells was sufficient to induce some ovarian development (Thorne, 1995).

In contrast to the avian Z sex chromosome, the W is small and largely heterochromatic. Only a small number of genes have been mapped to the avian (chicken) W chromosome, and most have Z-linked homologues. There are few candidate female-determining genes that are unique to the W (Smith, 2007). The best candidate sex-determining gene on the avian W chromosome is *HINTW*, a *Hi*stidine triad *N*ucleo*t*ide binding protein,-*W* linked. (also known as *WPKCI* and *ASW*) (Hori *et al.,* 2000; O'Neill *et al.,* 2000). *HINTW* encodes an aberrant form of a nucleotide hydrolase enzyme (HINT). HINT proteins have endogenous adenosine 5' monophosphoramidase enzyme activity. However, unlike all other HINT proteins, the predicted *HINTW* gene product specifically lacks a functional catalytic domain. A bona fide *HINT* gene is present on the Z chromosome (*HINTZ*), and several lines of *in vitro* biochemical evidence indicate that translated HINTW protein can interfere with the function of the Z form (Pace and Brenner, 2003).

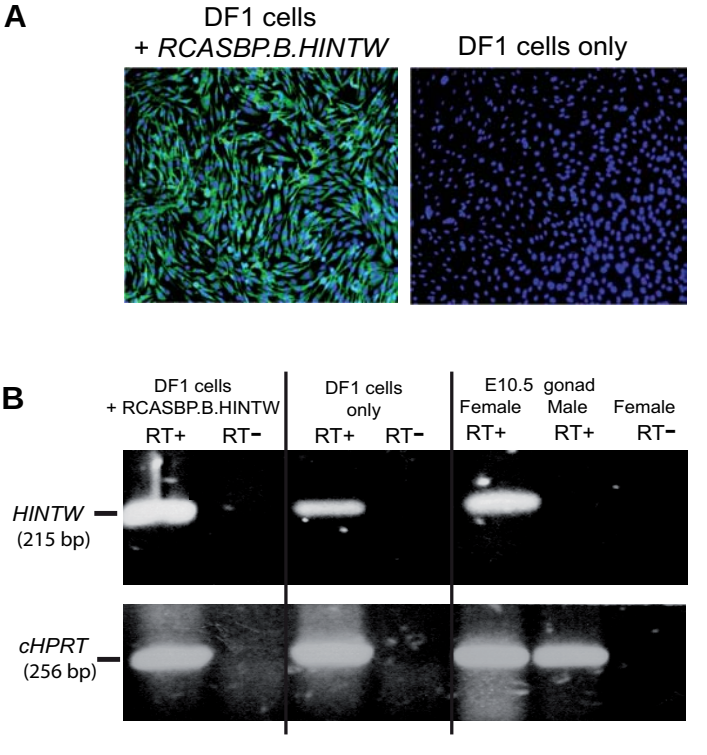


Fig. 2. Over-expression of *HINTW* in chicken DF1 cells. (A) *Immunofluorescent detection of viral-specific p27 antigen in DF1 cells infected with* RCASBP.B.HINTW. *No p27 staining if detectable in uninfected DF1 cells.* **(B)** *Mis-expression of* HINTW *in DF1 cells. Cells were uninfected or infected with* RCASBP.B.HINTW *and propagated for 10 days. RT-PCR analysis showed strong* HINTW *expression in infected cells, with a lower level of endogenous expression in uninfected DF1 cells. Gonadal cDNA from E10.5 female and male gonads served as positive and negative controls, respectively. Chicken* HPRT *was used as an internal control.*

HINTW is reiterated along the chicken W chromosome (Hori *et al.,* 2000), and the gene has been positively selected over evolution (Backstrom *et al.,* 2005). *HINTW* mRNA is widely expressed in the gonads and other tissues of female chicken embryos (Hori *et al.,* 2000; O'Neill *et al.,* 2000). While both *HINTW* and *HINTZ* are expressed in embryonic chicken gonads, *HINTW* is more strongly expressed (Hori *et al.,* 2000). Taken together, these data identify *HINTW* as a candidate sex-determining gene in birds (Smith and Sinclair, 2004).

Some lines of evidence do not support a universal role for *HINTW* in avian sex determination. The gene appears to be absent from ratites (flightless birds) (O'Neill *et al.,* 2000). In addition, *HINTW* mRNA is expressed throughout the body in ZW (female) chicken embryos, from as early as day 2, and it is expressed in the urogenital system well before gonadal sex differentiation (Hori *et al.,* 2000).

Here we describe the mis-expression of *HINTW* in chicken embryos. To mimic the normal expression pattern of the gene in genetic females, *HINTW* was mis-expressed throughout the urogenital system and body of early developing chicken embryos (ZZ males and ZW females). *HINTW* mis-expression did not alter testicular or ovarian development. Therefore, we conclude that *HINTW* is unlikely to play a dominant role in avian sex determination.

Results

Over-expression of a positive control protein (eGFP) in chicken embryos

To firstly demonstrate global mis-expression of a positive control in chicken embryos, we infected day 0 blastoderms with concentrated RCASBP.B.eGFP. Incubation was then allowed to proceed for four, five or nine days. In most embryos examined at these timepoints, global eGFP expression was achieved. (Fig. 1A). In negative control embryos not injected with virus, no eGFP was detectable. This preliminary experiment demonstrated the efficacy of using RCASBP viral vectors to deliver ubiquitous gene (and protein) expression in avian embryos.

Production of HINTW-*expressing virus in DF1 cells*

Chicken fibroblastic DF1 cells were transfected with RCASBP.B.HINTW and the cells were cultured for ten days. After this time, an aliquot of cells showed 100% infection with virus, as determined by p27 viral antibody staining (Fig. 2A). As there is no available antibody against the predicted HINTW protein, mRNA expression in DF1 cells was confirmed by RT-PCR. Strong *HINTW* mRNA expression was observed in infected DF1 cells, in day 10.5 control female gonads and not in male gonads (negative control). Uninfected DF1 cells showed endogenous expression of *HINTW* (indicating that this cell line must be of female origin), but expression was not as high as in the infected cells (Fig. 2B). Active virus was harvested from these cells, concentrated and injected into day 0 blastoderms of freshly laid eggs.

Mis-expression of HINTW *in chicken embryos*

Of sixty day 0 blastoderms injected with RCASBP virus expressing *HINTW*, 41 (68%) were alive at the time of harvest (day 9), indicating that global *HINTW* mis-expression is not embryo lethal.

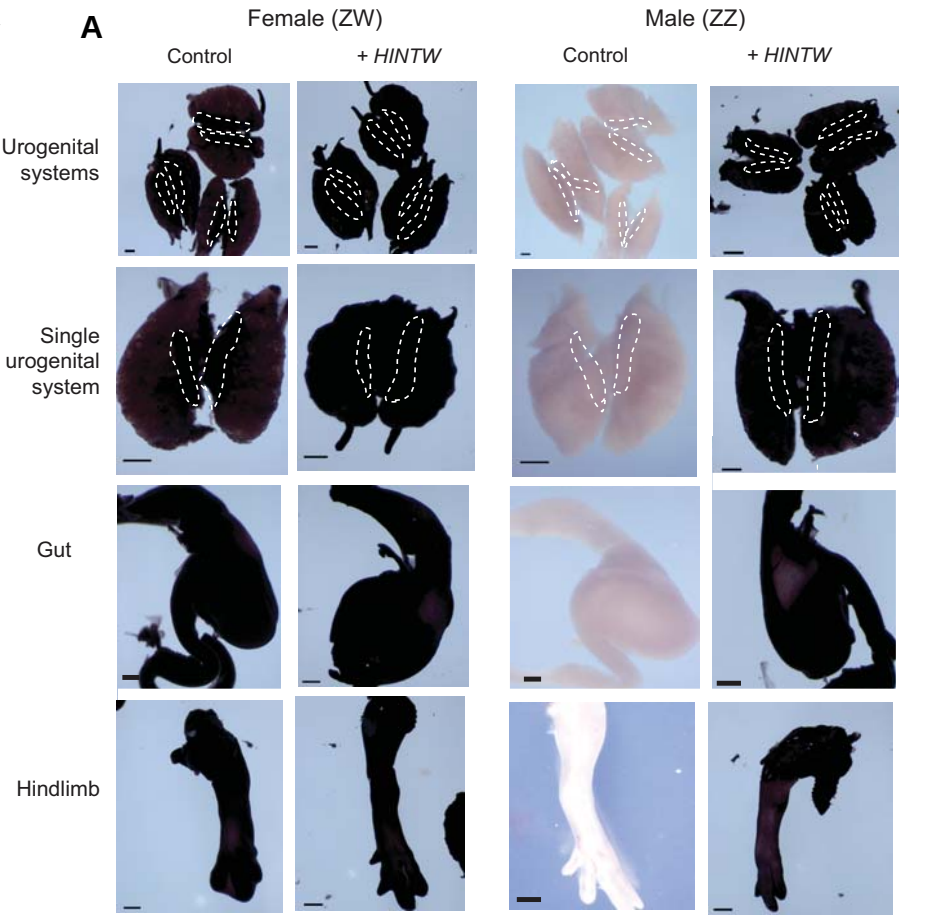
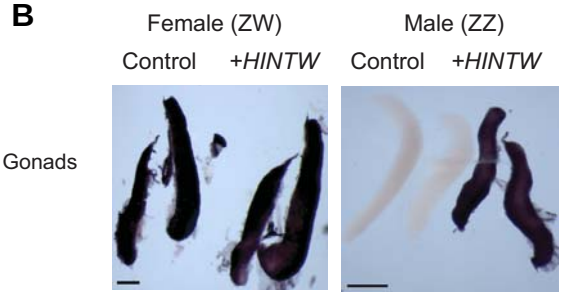


Fig. 3. Mis-expression of *HINTW* in female and male chicken embryos at embryonic day nine, assessed by whole mount *in situ* hybridisation. (A) *Whole mount* in situ *hybridisation of tissues taken from uninfected controls and embryos infected with* RCASBP.B.HINTW *at day 0. Control and infected females (ZW) show strong* HINTW *expression in the urogenital system, and in other tissues, such as the gut and hindlimb. Control male tissues (ZZ) show no expression, while infected males show strong expression in the urogenital sytem, gut and hindlimb, mimicking control female expression. Scale bar, 0.5 mm, except for hindimbs, where bar represents 1 mm.* **(B)** *Isolated gonads, showing strong* HINTW *expression in control and infected females (ZW), and in infected males (ZZ). Note the typical size asymmetry seen in the left (L) and right (R) female gonads, which is not seen in control or infected males.*



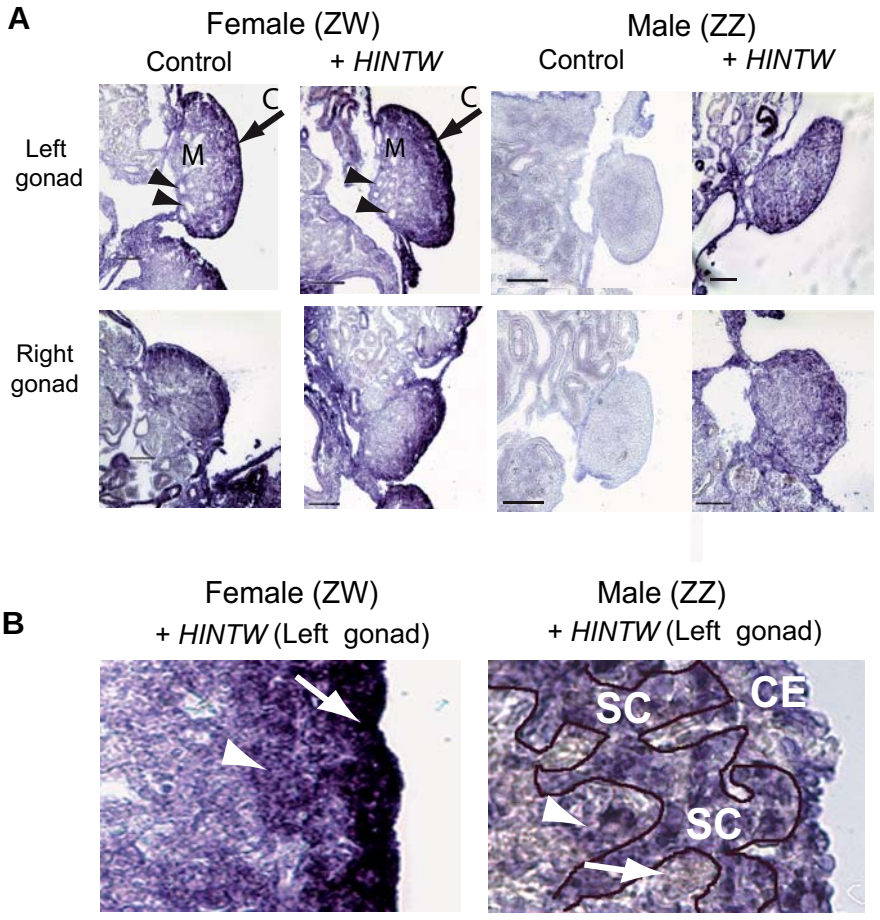


Fig. 4. *HINTW* mis-expression within day nine embryonic chicken gonads. *Sectioned whole mount tissues.* **(A)** *Low power magnification. In ZW embryos, Strong* HINTW *expression is detectable in both control and transgenic female left and right gonads. In the left gonad (ovary) expression is strong in the outer cortex (C), with lower expression in the underlying medulla (M). The medulla of the left gonad shows characteristic lacunae (arrowheads), in both control and transgenic embryos. In ZZ male embryos, no* HINTW *expression is detectable in either left or right gonads of controls, but strong expression is detectable in transgenic gonads.* **(B)** *High power magnification of gonads from transgenic embryos. In the left gonad of ZW females transgenic for* HINTW*, expression is strongest in the outer cortex (arrow), with a lower level of expression in the medulla (arrowhead). In both gonads of ZZ males transgenic for* HINTW*, expression is detectable in the outer coelomic epithelium (CE), in developing seminiferous cords (SC) and in surrounding interstitial cells (arrowhead). Some areas of the interstitium do not show* HINTW *mis-expression (arrow). Scale bar represents 100 μm.*

This is in agreement with the known expression profile of *HINTW* in female (ZW) embryos, in which expression is widespread from early stages. Embryos infected with control virus (RCASBP.B.eGFP) showed a similar level of survival, and global eGFP expression (including the gonads). RCASBP.B.eGFP served as a negative control for viral infection; gonads developed normally in embryos infected with RCASBP.B.eGFP (not shown). At the time of dissection (day 9), all embryos were genetically sexed by PCR. In control female (ZW) embryos not infected with virus, strong endogenous *HINTW* expression was detectable in the gonads and other tissues (gut and limbs) by whole mount *in situ* hybridisation. Female embryos (ZW) infected with virus also showed strong *HINTW* expression, throughout the gonads, mesonephric kidneys and in other tissues tested (heart, gut and limbs) (Fig. 3A). In these infected females, the detected *HINTW* expression represented endogenous and exogenous (virally delivered) expression. Indeed, expression appeared stronger (darker) in the infected females, compared to the uninfected controls. In control males (ZZ) not infected with virus, no *HINTW* expression was detected, as expected. In contrast, all ZZ genetic males infected with virus carrying *HINTW* (a total of fifteen embryos) showed robust *HINTW* expression in the gonads, mesonephric kidneys, and other tissues tested (gut and limb) (Fig 2A). The intensity of staining was similar to that seen in infected females. Some gonads were dissected away from the mesonephric kidneys and examined separately (Fig. 3B). Uninfected control females and transgenic females (ZW) showed strong *HINTW* expression in the gonads, and displayed the left-right size asymmetry typical of females at this stage (the left being larger than the right). Infected male gonads (ZZ) showed strong *HINTW* expression, with none detected in negative control male gonads. All gonads from male embryos infected with *RCASBP.B.HINTW* showed gross anatomy typical of paired testes, that is, "sausage-shaped" with no size asymmetry (Fig. 3B). This suggested that genetic males (ZZ) transgenic for *HINTW* were not sex-reversed. Gonads were over-stained in colour reaction and sectioned for further examination.

In ZW females embryos, both control gonads and those mis-expressing *HINTW* showed typical ovarian structure. The left gonad of both control and *HINTW* transgenic embryos exhibited strong *HINTW* staining in a well-developed outer cortex, with staining also present in the underlying medulla (Fig. 4A). At this stage of development, the left ovary has characteristic lacunae (spaces) in the medulla. The right gonad did not form a cortex, as expected, while *HINTW* expression was detectable throughout the medulla. In control males (ZZ), the gonads were testicular in appearance. Gonads of males infected with *HINTW* also appeared testicular, but with strong *HINTW* staining throughout (Fig. 4A). In left female gonads, high power magnification revealed strong *HINTW* mRNA expression in the ovarian cortex and a lower level of staining throughout the underlying medulla (stronger in the so-called juxtacortical medulla, immediately adjacent to the cortex) (Fig. 3). High power magnification of transgenic ZZ male gonads showed strong *HINTW* expression within seminiferous cords, and in areas between the cords (Fig. 4).

Gonadal histology and expression of marker proteins

In a second experiment, thirty blastoderms were infected with RCASBP virus carrying *HINTW*, and harvested at day nine. Some tissues were taken for quantitative RT-PCR, while others were taken for histology. Normal control gonads from E6.5 and 8.5

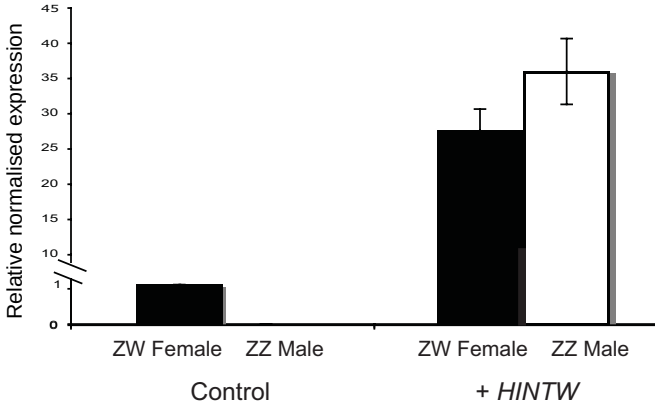


Fig. 5. Mis-expression of *HINTW* in day nine chicken embryonic gonads, as assessed by real time RT-PCR. *Control 8.5 female (ZW) gonads show* HINTW *expression (normalised to 1), and control ZZ males show no expression. Gonads from both ZW female and ZZ male embryos infected with* RCASBP.B.HINTW *show robust expression.*

embryos showed female-specific *HINTW* expression, with no expression in ZZ males, as expected. In contrast, a robust *HINTW* mRNA expression was detected in both ZW female and ZZ male gonads taken from embryos infected with virus carrying *HINTW*. (Fig. 5). The gonads of ZW female and ZZ male embryos mis-expressing *HINTW* showed normal gonadal histology at day nine (Fig. 6). In ZW females infected with RCASBP virus carrying *HINTW*, normal asymmetry was seen, characterised by a well-developed left ovary and a regressing right gonad (Fig. 6). The left ovary exhibited a typical ovarian structure, with thickened outer cortex and an underlying medulla with lacunae (spaces). The right gonad lacked a well-developed cortex, as in normal control females (Fig. 6). In ZZ males mis-expressing *HINTW*, normal testicular morphology was evident, characterised by well-developed seminiferous cords in both gonads (Fig. 6).

In both ZW female and ZZ male infected embryos, strong p27 immunostaining confirmed viral expression in the gonads (Fig. 7). No p27 staining was detected in negative control tissues from uninfected embryos. In ZW females mis-expressing *HINTW*, a normal pattern of aromatase enzyme expression was found (Fig. 7). Aromatase is a key marker of ovarian differentiation in birds, expressed in the medulla of both left and right gonads. While aromatase is expressed female-specifically, SOX9 is expressed male-specifically. In ZZ males mis-expressing *HINTW*, a normal pattern of SOX9 protein expression was detected. As in control gonads, Chicken Vasa Homologue (CVH) staining revealed a typical pattern of germ cell distribution in both sexes. In ZW female embryos mis-expressing *HINTW*, germ cells had accumulated in the left ovarian cortex, as in controls. ZZ males mis-expressing *HINTW* showed a typical male-specific distribution of germ cells in the developing seminiferous cords (Fig 7). Altogether, the histology and immunofluorescence indicated normal gonadal development in both sexes mis-expressing *HINTW*.

Discussion

In normal genetic female chicken embryos (ZW), *HINTW* is strongly expressed throughout the body. Expression is detectable as early as day 2.5 (Yamada *et al.,* 2004), and it persists in the developing urogenital system, nervous system, somites and limbs. In the embryonic gonads, the gene is expressed at all stages that have been examined, from the time of gonadal formation (day 3), throughout sexual differentiation (from day 6), and up to hatching (Hori *et al.,* 2000). In the experiments described here, we aimed to mimic this widespread female pattern of expression by delivering *HINTW* into male embryos from the earliest stage possible (day 0 blastoderms). Infection of day 0 blastoderms resulted in widespread *HINTW* expression in genetic males, which lack the endogenous gene. Despite this widespread expression, including strong expression in the gonads, testes developed normally. These results provide genetic evidence against the hypothesis that *HINTW* plays a role in avian

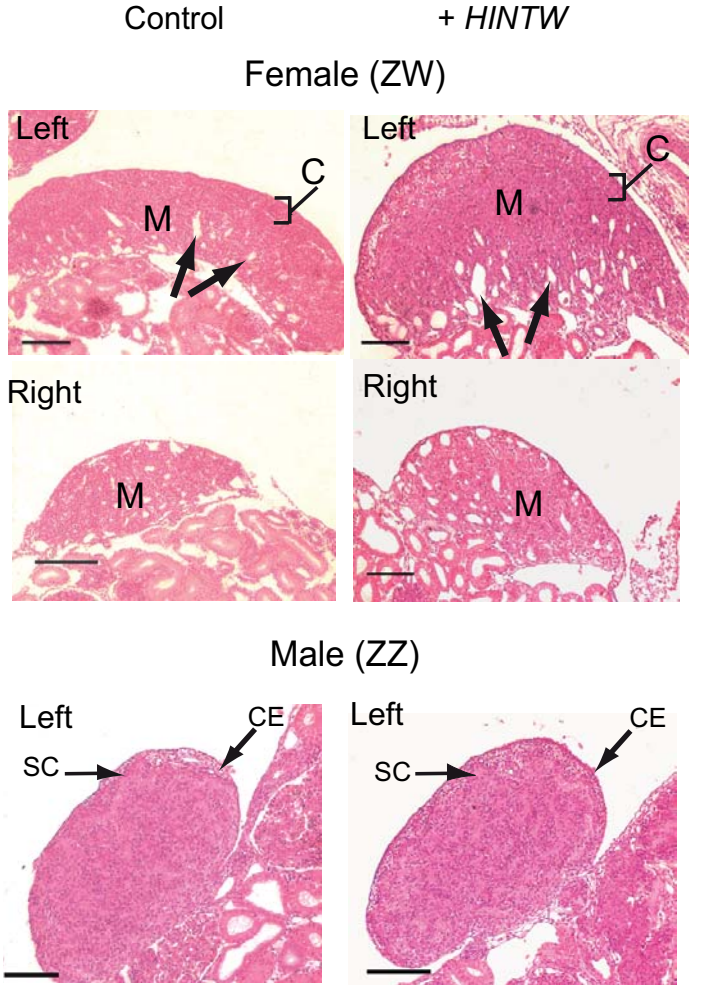


Fig. 6. Normal gonadal histology of day nine chicken embryos mis-expressing *HINTW*. *Haematoxylin and eosin staining. Normal asymmetric ovarian development is seen in ZW control females and in ZW females mis-expressing* HINTW. *The left gonad has a thickened outer cortex (C) and fragmented medulla (M), while the right gonad lacks a cortex. The left ovary had developed characteristic lacunae (arrows). Normal bilateral testicular morphology is seen in control ZZ males and in ZZ males mis-expressing* HINTW, *characterised by developing seminiferous cords (SC), surrounded by interstitium and an thin out coelomic epithelial layer (CE). For males, the left gonad only is shown. Scale bar, 100 μm.*

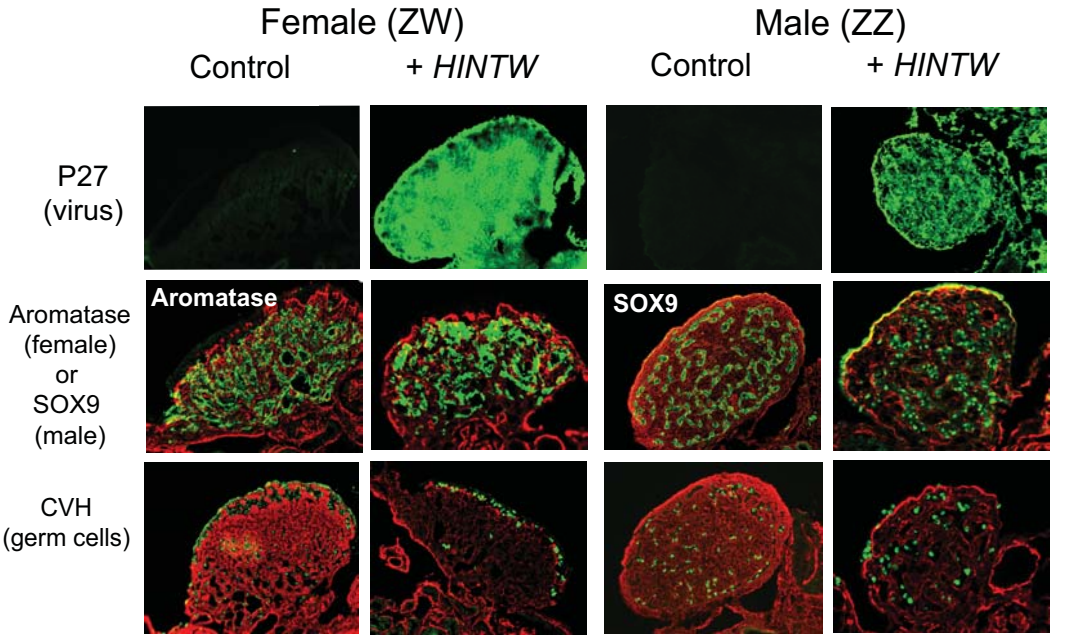


Fig. 7. Expression of marker proteins in day nine embryonic chicken gonads mis-expressing *HINTW*. *Immunofluorescent detection, shown in green (counterstained with anti-fibronectin in red). Strong expression of the viral p27 protein is detectable in gonads of embryos infected with RCASBP.B.HINTW, but not in uninfected controls. In ZW females mis-expressing* HINTW, *the female-specific marker, Aromatase, is expressed normally in the gonadal medulla. In ZZ males mis-expressing* HINTW, *the male marker, SOX9, is expressed normally in Sertoli cells of the seminiferous cords. The germ cell marker, CVH, delineates a normal germ cell distribution pattern in ZW females and ZZ males mis-expressing* HINTW. *In females, CVH+ germ cells accumulate in the outer cortex, as in control females, while, in males, they become localised within seminiferous cords.*

(chicken) sex determination.

The positive control (eGFP) demonstrsted that the RCASBP viral vector is an efficient vehicle for delivering global expression of a protein in chicken embryos. The lack of a phenotype observed in embryos infected with RCASBP.B.HINTW is therefore unlikely to be due to a lack of transgene expression. It is possible that the level of *HINTW* mis-expression delivered by the RCASBP virus was not sufficient to affect gonadal development. However, this is unlikely, because the viral LTR promoter is very strong, driving high-level expression, and quantitative RT-CPR analysis showed robust mis-expression of *HINTW* mRNA in the gonads of infected embryos (at least 25 fold higher than that of normal ZW females). Furthermore, mis-expression in the appropriate cell types was achieved (in the medullary cords and the surface epithelium of gonads; Fig. 4), precluding the possibility of cell type restricted mis-expression. Given that embryos were infected at the blastoderm stage, it is anticipated that essentially global *HINTW* expression would be achieved, as supported by the whole mount results (Fig. 3). Furthermore, the embryonic (mesonephric) kidneys of infected embryos were always strongly positive for *HINTW* expression. This points to strong infection of the kidney primordia (derived from intermediate mesoderm) very early in development. The RCASBP virus integrates permanently into the genome of each cell, passing vertically to daughter cells as well as horizontally to neighbouring cells. This ensures strong and stable mis-expression of the transgene. Therefore, since the gonads derive from the mesonephric kidneys, they are also expected to show strong transgene expression. This was indeed the case.

HINTW belongs to a family of genes that encode nucleotide hydrolase enzymes. These enzymes are nucleotide-binding factors that have intrinsic adenosine 5'-monophosphoramidase activity. However, the predicted HINTW protein lacks the key catalytic motif common to all other HINT enzymes. Several *in vitro* biochemical studies have shown that over-expression of the predicted HINTW protein blocks the function of the Z-linked homologue, HINTZ (Moriyama *et al.,* 2006; Pace and Brenner, 2003; Parks *et al.,* 2004). *HINTZ* encodes a *bona fide* HINT protein that has adenosine monophosphoramidase activity, but it can heterodimerise with HINTW *in vitro*, causing a dramatic reduction in its function. It has been hypothesised that HINTW could act as a dominant negative in avian sex determination, blocking the function of HINTZ, which would be testis-promoting (Hori *et al.,* 2000; O'Neill *et al.,* 2000; Pace and Brenner, 2003). However, an endogenous HINTW protein has not yet been demonstrated; there is no evidence that the *HINTW* transcript is in fact translated. We raised an antibody against the deduced HINTW protein, but it failed to detect any endogenous HINTW protein. It therefore remains possible that, under normal conditions, *HINTW* is transcribed but not translated. In the *HINTW* mis-expression experiments conducted here, the transgene was transcribed, as expected, and it is also expected to have been translated. If translated, HINTW may have a role in embryonic development that is not related to gonadal sex differentiation *per se*.

The data presented here indicate that *HINTW* does not have a dominant role in avian sex determination. However, it may have a role in the female pathway that is overridden by the male pathway in ZZ embryos. Thus, in ZZ embryos mis-expressing *HINTW*, the male pathway may antagonise HINTW function such that testis development can still occur. For example, a higher dose of the Z-linked candidate male factor, DMRT1, may override HINTW in ZZ embryos. Our study does indicate, however, that HINTW is unlikely to operate as a *dominant* factor in avian sex determination. Further studies are now required to address the possible requirement of *HINTW* for female development. Specifically, knock down of endogenous *HINTW* expression in ZW female embryos would address this issue. Over-expression of short interfering RNAs could be used in this instance, as it has recently been shown that the RCAS viral

vector can be used for RNA interference (Harpavat and Cepko, 2006).

The study reported here is the first to directly test a candidate avian sex-determining gene by mis-expression in the embryonic gonads. The results suggest that *HINTW* does not play a role in avian sex determination, at least not via the dominant mechanism postulated. This implies that another, as yet unidentified, W-linked gene may be ovary-determining in birds. The alternative hypothesis also remains viable, that is, sex determination via a Z dosage mechanism.

Materials and Methods

Preparation of RCASBP.B.HINTW *viral vector*

For mis-expression of *HINTW*, we used the avian retroviral vector, RCASBP.B (Replication-Competent Avian sarcoma-leukosis virus, long terminal repeat (LTR) with a'Splice acceptor, Bryan Polymerase, subtype B). RCASBP.B proviral DNA and associated plasmids were a gift from Professor Cliff Tabin (Department of Genetics, Harvard Medical School). Specific forward and reverse primers were designed to amplify the 393 base pair *HINTW* open reading frame from embryonic chicken gonads *HINTW.*ORF.
Forward: 5'-AGTCCATGGCCGGCGGGATCGTTAGG-3',
Reverse: 5'-TACCGAATTCTTAGCCAGGAGGCTGGCCCAAC-3'). The forward primer included a (natural) *Nco*1 site and the reverse primer included an artificial *EcoR*1 site for cloning into the shuttle plasmid, pSlax13 (underlined above) (Logan and Tabin, 1998). The ORF was amplified with high fidelity Proofstart DNA polymerase according to the manufacturer's instructions (QIAGEN), and cloned into Nco1+EcoR1 digested *pSLAX13* shuttle vector. The identity of the insert was confirmed by sequencing. The *pSLAX13-HINTW* plasmid DNA was then digested with *Cla*1, the insert gel purified and cloned into *Cla*1-digested RCASBP.B. Since cloning into RCASBP is non-directional, the correct orientation was confirmed by PCR with a 5' RCASBP primer together with an internal HINTW 3' (reverse) primer. High purity, endotoxin-free *RCASBP.B.HINTW* DNA was prepared from DH5α cells and 10μg was used to transfect the DF1 chicken fibroblastic cell line. Lipofectamine 2000 (Invitrogen) was used for transfection. Cells were grown and passaged for 10 days prior to testing for viral expression using an antibody against the viral protein, p27. For p27 staining, an aliquot of infected versus non-infected DF1 cells were grown in a 24 well plate, fixed in -20°C methanol for 10 minutes, rinsed in PBS, blocked in 2% BSA/PBS, then incubated overnight with rabbit anti-p27 antibody (1:1000; Charles River Laboratories). Following washes in PBS, Alexa fluor 488 goat anti-rabbit antibody was added for one hour (1:1000; Molecular Probes). DAPI was added to visualise cells prior to mounting in Fluorosave (Calbiochem). Only the infected DF1 cells showed global p27 expression. To test for *HINTW* expression, RNA was extracted from uninfected DF1 cells and from cells infected with'*RCASBP.B. HINTW*. Total RNA was treated with DNase (Ambion DNA-free kit), and RT-PCR analysis was then performed for *HINTW* mRNA expression, using the primers,
For: 5'-CGTTGTGGACGAGGAGTGCC-3';
Rev: 5'-TGAGGGTGGGTATCTCACCAGCC-3'; 215bp cDNA product). Chicken HPRT controls were also included:
For: 5'-TGGGCTCATCATGGACAGGAC-3';
Rev: 5'-GGTTGAGAGGTCATCCCCACC-3'; 256bp cDNA product).

Active virus was harvested and concentrated from DF1 cells as described previously (Logan and Tabin, 1998; Morgan and Fekete, 1996). Briefly, culture medium was taken from 6 large flasks of DF1 cells infected with *RCASBP.B.HINTW*. Medium was collected over 3 days, ultracentrifuged, resuspended in a small volume (100 -300 μL), aliquotted in 20 μL volumes and stored at -80°C. Two types of negative controls were included. For negative controls, we used RCASBP.B.EGFP, transfected into DF1 cells and prepared as for RCASBP.B.HINTW (Smith *et al.,* 2008). Another set of controls comprised uninfected embryos.

Infection of embryos with RCASBP.B.HINTW

Viral titre was firstly assessed by adding serial dilutions of concentrated RCASBP.B.HINTW virus to uninfected DF1 cells for 48 hours and then staining for p27 expression. Isolated colonies of p27 expressing cells were counted and titres of $1x\ 10^8 – 1\ x\ 10^9$ Infectious Units/mL were obtained. This is within the range considered suitable for robust infection of embryos (Logan and Tabin, 1998). Fertile eggs susceptible to RCASBP virus were obtained from SPAFAS (Woodend, Victoria). Concentrated virus containing 0.025% Fast Green tracking dye was injected into the subgerminal cavity of day 0 blastoderms. Approximately 2-3 μL were injected per embryo. Eggs were re-sealed and incubated horizontally with rocking at 37.8°C until day 3. Eggs were then placed upright, some albumen was removed and incubation resumed without rocking until day 9. (Gonadal sex differentiation begins on day 6 and is normally advanced by day 9.) Embryo survival from day 0 to day 3 was over 90% and overall survival from day 0 to day 9 was 68%. Ninety eggs in total were injected. For negative controls, RCASBP.B.EGFP was injected into 40 eggs. This resulted in global eGFP expression (including gonadal expression) that was compatible with sexual differentiation. Hence, infection with the RCASBP virus itself did not perturb normal male and female development. Survival rates of embryos infected with RCASBP.B.eGFP were similar to those obtained for RCASBP.B.HINTW infections. A second group of negative controls comprised embryos that were not infected with any viruses.

PCR sexing

Infected and controls embryos were dissected at day 9 (developmental stage 35) (Hamburger and Hamilton, 1951). For genetic sexing of embryos, a small piece of limb tissue was digested in PCR compatible Proteinase K buffer (McClive and Sinclair, 2001) and the genomic DNA used for rapid PCR sexing (Clinton *et al.,* 2001). By this method, only females show a W-linked (female-specific) *Xho*1 band. Amplification of *18S rRNA* serves as an internal control.

HINTW quantitative Reverse Transcription-PCR (qRT-PCR)

RNA was extracted from paired gonads derived form negative control embryos and those infected with RCASBP.B.HINTW. Approximately five pairs of gonads were used for each sex at each stage. Total RNA was subjected to DNase treatment to remove contaminating genomic DNA using the Ambion DNA-free kit. For each sample, 400-1000ng DNased RNA was reverse transcribed using Superscript III™ (InVitrogen) and a mixture of oligo-dT and random hexamers as primers. One μL of each RT reaction was used for each PCR reaction. Primers spanning exon-exon boundaries were designed, and care was taken to avoid amplification of the related *HINTZ* sequence. Primers were used and together with the Universal Probe Library (UPL) system and Faststart Probe Master mix with ROX (Roche). Real time PCR was performed on an ABI 7900 HT instrument. Samples were run in triplicate and experiments performed at least twice. All samples were normalised against *cHPRT* using the comparative CT method (ΐΐCT). HPRT (which is autosomal in chicken) was found to be a stably expressed during chicken gonadal development. Cycle parameters and sequences for all qRT-PCR primer/UPL probe set combinations are included in Supplementary Table 1. For all qRT-PCR data presented here, no template controls or RT- samples exhibited no amplification. In addition, each amplification set was performed with standard curves to confirm primer/probe efficiency, > 90%).

Whole mount in situ *hybridisation*

Urogenital and other tissues were dissected from day 9 infected and non-infected control embryos. Whole mount *in situ* hybridisation was used to assess *HINTW* mis-expression. Tissues were fixed overnight at

4°C in RNAse-free 4% paraformaldehyde/PBS, washed in cold PBS containing 0.1% Triton X-100 (PBTX), and dehydrated through a series of methanol solutions, ending with 100% methanol. Tissues were stored overnight at -20°C prior to rehydration through a descending methanol series and into PBTX. Whole mount *in situ* hybridisation for *HINTW* mRNA expression was carried out as described previously (O'Neill *et al.,* 2000a; Smith *et al.,* 1999). Briefly, tissues were rehydrated, digested for 45 minutes in 10μg/mL proteinase K in PBTX, pre-hybridised overnight at 65°C, then hybridised under the same conditions in the presence of a digoxygenin-labelled *HINTW*-specific antisense riboprobe. This probe was generated from gonadal cDNA and has been described previously [31]. Following stringency washes in 2x SSC and 0.2x SSC, tissues were blocked in PBTX containing BSA and sheep serum, followed by incubation in PBTX/BSA/sheep serum containing alkaline phosphatase conjugated DIG antibody (Roche) (overnight at 4°C). Tissues were then extensively washed in Tris buffer/ BSA (overnight). Colour reaction (BCIP/NBT) was added in Tris-based buffer for 1.5 hours at room temperature. The reaction was stopped after a deep purple precipitate became visible. Sense controls did not show any colour reaction. Following photography, some tissues were then over-stained by immersion in colour reagent for a further 48 hours, prior to cryoprotection in 30% sucrose/PBS and embedding in OCT for tissue sectioning. Fourteen to eighteen μm frozen sections were taken of over-stained tissues, mounted in Aquamount and photographed.

Gonadal histology and analysis of gonadal marker proteins

In a second experiment, 30 embryos were infected with *RCASBP.B.HINTW* at day 0, as described above, dissected at day 9 and urogenital systems were fixed for standard H & E staining or immunofluorescence. Paired urogenital systems were cut in half transversely, and one half taken for histology, while the other half was taken for immunofluorescent detection of gonadal marker proteins. For histology, tissues were fixed overnight at room temperature in Bouin's fluid, dehydrated in 70% ethanol and processed into paraffin blocks. Six micron sections were stained with haematoxylin and eosin. For immunofluorescence, tissues were fixed briefly in 4% PFA in PBS, cryoprotected overnight at 4°C in 30% sucrose/PBS, immersed in OCT embedding compound and snap frozen in isopentane pre-cooled in liquid nitrogen. Some tissues were directly snap frozen without fixation. Ten micron frozen sections were either collected in PBS or post-fixed for 10 minutes in methanol at -20°C (for p27 viral staining only). Regardless of fixation method, tissues were then blocked in 2% BSA/PBS (2 hours), and incubated with primary antibodies diluted in 0.1% Triton-X 100/ 1% BSA/PBS (overnight at 4°C). Most primary antibodies were raised in house (Smith *et al.,* 2003), rabbit anti-chicken SOX9 (1:6000), rabbit anti-chicken Aromatase (1:5000) and rabbit anti-Chicken CVH Homologue (CVH) (1:6000). A mouse anti-chicken fibronectin antibody was also used to delineate overall gonadal structure (Serotec, 1:200). Secondary antibodies were Alexafluor 488 donkey anti-rabbit IgG (green) (used at 1:1000) and Alexafluor 594 donkey anti-mouse IgG (red) (used at 1:1500) (Molecular Probes, Invitrogen).

Acknowledgements

The authors thank Professor Cliff Tabin, Harvard Medical School, for the RCASBP.B viral vectors. The work was supported by an Australian Research Council (ARC) grant awarded to CAS and AHS.

References

ARIT, D., BENSCH, S., HANSSON, B., HASSELQUIST, D. AND WESTERDAHL, H. (2004) Observation of a ZZW female in a natural population: implications for avian sex determination. *Proc Biol Sci* 271 Suppl 4: S249-S251.

BACKSTROM, N., CEPLITIS, H., BERLIN, S. AND ELLEGREN, H. (2005) Gene conversion drives the evolution of HINTW, an ampliconic gene on the female-specific avian W chromosome. *Mol Biol Evol* 22: 1992-1999.

BERTA, P., HAWKINS, J.R., SINCLAIR, A.H., TAYLOR, A., GRIFFITHS, B.L., GOODFELLOW, P.N. AND FELLOUS, M. (1990) Genetic evidence equating SRY and the testis-determining factor. *Nature* 348: 448-450.

CLINTON, M., HAINES, L., BELLOIR, B. AND MCBRIDE, D. (2001) Sexing chick embryos: a rapid and simple protocol. *Br Poult Sci* 42: 134-138.

ELLEGREN, H. (2000) Evolution of the avian sex chromosomes and their role in sex determination. *Trends Ecol Evol* 15: 188-192.

ELLEGREN, H., HULTIN-ROSENBERG, L., BRUNSTROM, B., DENCKER, L., KULTIMA, K. AND SCHOLZ, B. (2007) Faced with inequality: chicken do not have a general dosage compensation of sex-linked genes. *BMC Biol* 5: 40.

GRAVES, J.A. (2003) Sex and death in birds: a model of dosage compensation that predicts lethality of sex chromosome aneuploids. *Cytogenet Genome Res* 101: 278-282.

HAMBURGER, V. AND HAMILTON, H.L. (1951) A series of normal stages in the development of the chick embryo. *J Morph* 88: 49-92.

HARPAVAT, S. AND CEPKO, C.L. (2006) RCAS-RNAi: a loss-of-function method for the developing chick retina. *BMC Dev Biol* 6: 2.

HORI, T., ASAKAWA, S., ITOH, Y., SHIMIZU, N. AND MIZUNO, S. (2000) Wpkci, encoding an altered form of PKCI, is conserved widely on the avian W chromosome and expressed in early female embryos: implication of its role in female sex determination. *Mol Biol Cell* 11: 3645-3660.

ITOH, Y., MELAMED, E., YANG, X., KAMPF, K., WANG, S., YEHYA, N., VAN NAS, A., REPLOGLE, K., BAND, M.R., CLAYTON, D.F., SCHADT, E.E., LUSIS, A.J. AND ARNOLD, A.P. (2007) Dosage compensation is less effective in birds than in mammals. *J Biol* 6: 2.

KIM, S., BARDWELL, V.J. AND ZARKOWER, D. (2007) Cell type-autonomous and non-autonomous requirements for Dmrt1 in postnatal testis differentiation. *Dev Biol* 307: 314-327.

KOOPMAN, P., GUBBAY, J., VIVIAN, N., GOODFELLOW, P. AND LOVELL-BADGE, R. (1991) Male development of chromosomally female mice transgenic for Sry. *Nature* 351: 117-121.

LIN, M., THORNE, M.H., MARTIN, I.C.A., SHELDON, B.L. AND JONES, R.C. (1995) Development of the gonads in the triploid (ZZW and ZZZ) fowl, *Gallus domesticus*, and comparison with normal diploid males (ZZ) and females (ZW). *Reprod Fert Dev* 7: 1185-1197.

LOGAN, M. AND TABIN, C. (1998) Targeted gene misexpression in chick limb buds using avian replication-competent retroviruses. *Methods* 14: 407-420.

MATSUDA, M., NAGAHAMA, Y., SHINOMIYA, A., SATO, T., MATSUDA, C., KOBAYASHI, T., MORREY, C.E., SHIBATA, N., ASAKAWA, S., SHIMIZU, N., HORI, H., HAMAGUCHI, S. AND SAKAIZUMI, M. (2002) DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature* 417: 559-563.

MATSUDA, M., SHINOMIYA, A., KINOSHITA, M., SUZUKI, A., KOBAYASHI, T., PAUL-PRASANTH, B., LAU, E.L., HAMAGUCHI, S., SAKAIZUMI, M. AND NAGAHAMA, Y. (2007) DMY gene induces male development in genetically female (XX) medaka fish. *Proc Natl Acad Sci USA* 104: 3865-3870.

MCCLIVE, P.J. AND SINCLAIR, A.H. (2001) Rapid DNA extraction and PCR-sexing of mouse embryos. *Mol Reprod Dev* 60: 225-226.

MELAMED, E. AND ARNOLD, A.P. (2007) Regional differences in dosage compensation on the chicken Z chromosome. *Genome Biol* 8: R202.

MORGAN, B.A. AND FEKETE, D.M. (1996) Manipulating gene expression with replication-competent retroviruses. *Methods Cell Biol* 51: 185-218.

MORIYAMA, S., OGIHARA, J., KATO, J., HORI, T. AND MIZUNO, S. (2006) PKCI-W forms a heterodimer with PKCI-Z and inhibits the biological activities of PKCI-Z in vitro, supporting the predicted role of PKCI-W in sex determination in birds. *J Biochem* 139: 91-97.

MURPHY, M.W., ZARKOWER, D. AND BARDWELL, V.J. (2007) Vertebrate DM domain proteins bind similar DNA sequences and can heterodimerize on DNA. *BMC Mol Biol* 8: 58.

NANDA, I., ZEND-AJUSCH, E., SHAN, Z., GRUTZNER, F., SCHARTL, M., BURT, D.W., KOEHLER, M., FOWLER, V.M., GOODWIN, G., SCHNEIDER, W.J., MIZUNO, S., DECHANT, G., HAAF, T. AND SCHMID, M. (2000) Conserved synteny between the chicken Z sex chromosome and human chromosome 9 includes the male regulatory gene DMRT1: a comparative (re)view on avian sex determination. *Cytogenetics & Cell Genetics* 89: 67-78.

O'NEILL, M., BINDER, M., SMITH, C., ANDREWS, J., REED, K., SMITH, M., MILLAR, C., LAMBERT, D. AND SINCLAIR, A. (2000) ASW: a gene with conserved avian W-linkage and female specific expression in chick embryonic gonad. *Development Genes & Evolution* 210: 243-249.

OTTOLENGHI, C., PELOSI, E., TRAN, J., COLOMBINO, M., DOUGLASS, E., NEDOREZOV, T., CAO, A., FORABOSCO, A. AND SCHLESSINGER, D. (2007) Loss of Wnt4 and Foxl2 leads to female-to-male sex reversal extending to germ cells. *Hum Mol Genet* 16: 2795-2804.

PACE, H.C. AND BRENNER, C. (2003) Feminizing chicks: a model for avian sex determination based on titration of Hint enzyme activity and the predicted structure of an Asw-Hint heterodimer. *Genome Biol* 4: R18.

PARKS, K.P., SEIDLE, H., WRIGHT, N., SPERRY, J.B., BIEGANOWSKI, P., HOWITZ, K., WRIGHT, D.L. AND BRENNER, C. (2004) Altered specificity of Hint-W123Q supports a role for Hint inhibition by ASW in avian sex determination. *Physiol Genomics* 20: 12-14.

PARMA, P., RADI, O., VIDAL, V., CHABOISSIER, M.C., DELLAMBRA, E., VALENTINI, S., GUERRA, L., SCHEDL, A. AND CAMERINO, G. (2006) R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nat Genet* 38: 1304-1309.

RAYMOND, C.S., KETTLEWELL, J.R., HIRSCH, B., BARDWELL, V.J. AND ZARKOWER, D. (1999) Expression of Dmrt1 in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. *Dev Biol* 215: 208-220.

RAYMOND, C.S., MURPHY, M.W., O'SULLIVAN, M.G., BARDWELL, V.J. AND ZARKOWER, D. (2000) Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev* 14: 2587-2595.

SHAN, Z., NANDA, I., WANG, Y., SCHMID, M., VORTKAMP, A. AND HAAF, T. (2000) Sex-specific expression of an evolutionarily conserved male regulatory gene, DMRT1, in birds. *Cytogenetics & Cell Genetics* 89: 252-257.

SINCLAIR, A.H., BERTA, P., PALMER, M.S., HAWKINS, J.R., GRIFFITHS, B.L., SMITH, M.J., FOSTER, J.W., FRISCHAUF, A.M., LOVELL-BADGE, R. AND GOODFELLOW, P.N. (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* 346: 240-244.

SMITH, C., ROESZLER, K. AND SINCLAIR, A. (2008) Ubituitous GFP epxresion in a single generation of chicken embryos using the avian retroviral vector, RCASBP. *Dev Biol* submitted.

SMITH, C.A. (2007) Avian sex determination: HINTS from the W sex chromosome? *Sexual Development*.

SMITH, C.A., KATZ, M. AND SINCLAIR, A.H. (2003) DMRT1 is upregulated in the gonads during female-to-male sex reversal in ZW chicken embryos. *Biol Reprod* 68: 560-570.

SMITH, C.A., ROESZLER, K.N., HUDSON, Q.J. AND SINCLAIR, A.H. (2007) Avian sex determination: what, when and where? Cytogenet *Genome Res* 117: 165-173.

SMITH, C.A. AND SINCLAIR, A.H. (2004) Sex determination: insights from the chicken. *Bioessays* 26: 120-132.

SMITH, C.A., SMITH, M.J. AND SINCLAIR, A.H. (1999) Expression of chicken steroidogenic factor-1 during gonadal sex differentiation. *Gen Comp Endocrinol* 113: 187-196.

STIGLEC, R., EZAZ, T. AND GRAVES, J.A. (2007) A new look at the evolution of avian sex chromosomes. *Cytogenet Genome Res* 117: 103-109.

THORNE, M., H. (1995) Genetics of Poultry Reproduction. In Hunton, P. (ed.), Poultry Production, Elsevier, Amsterdam, pp. 411-434.

THORNE, M.H. (1997) A review of sex determination and differentiation in poultry. In, Proceedings of Australian Poultry Science SymposiumVol. 9, pp. 1-7.

THORNE, M.H. AND SHELDON, B.L. (1991) Cytological evidence of maternal meiotic errors in a line of chickens with a high incidence of triploidy. *Cytogenet Cell Genet* 57: 206-210.

WILHELM, D., PALMER, S. AND KOOPMAN, P. (2007) Sex determination and gonadal development in mammals. *Physiol Rev* 87: 1-28.

YAMADA, D., KOYAMA, Y., KOMATSUBARA, M., URABE, M., MORI, M., HASHIMOTO, Y., NII, R., KOBAYASHI, M., NAKAMOTO, A., OGIHARA, J., KATO, J. AND MIZUNO, S. (2004) Comprehensive search for chicken W chromosome-linked genes expressed in early female embryos from the female-minus-male subtracted cDNA macroarray. *Chromosome Res* 12: 741-754.

YOSHIMOTO, S., OKADA, E., UMEMOTO, H., TAMURA, K., UNO, Y., NISHIDA-UMEHARA, C., MATSUDA, Y., TAKAMATSU, N., SHIBA, T. AND ITO, M. (2008) A W-linked DM-domain gene, DM-W, participates in primary ovary development in Xenopus laevis. *Proc Natl Acad Sci USA* 105: 2469-2474.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

See our recent Special Issue ***Fertilization***, in honor of David L. Garbers and edited by Paul M. Wassarman and Victor D. Vacquier at: http://www.ijdb.ehu.es/web/contents.php?vol=52&issue=5-6

Sex determining mechanisms in insects
Lucas Sanchez
Int. J. Dev. Biol. (2008) 52: 837 - 856.

Aard is specifically up-regulated in Sertoli cells during mouse testis differentiation
Terje Svingen, Annemiek Beverdam, Pali Verma, Dagmar Wilhelm and Peter Koopman
Int. J. Dev. Biol. (2007) 51: 255-258

Gametophyte interaction and sexual reproduction: how plants make a zygote
Leonor C. Boavida, Ana Maria Vieira, Jörg D. Becker and José A. Feijó
Int. J. Dev. Biol. (2005) 49: 615-632

Molecular markers for germ cell differentiation in the demosponge Suberites domuncula
Sanja Perovic-Ottstadt, Helena Cetkovic, Vera Gamulin, Heinz C. Schroder, Klaus Kropf, Claire Moss, Michael Korzhev, Barbel Diehl-Seifert, Isabel M. Muller and Werner E.G. Muller
Int. J. Dev. Biol. (2004) 48: 293-305

Developmental biology for undergraduate students at the University of Palermo, Italy.
Giovanni Giudice and Karoly Onorato
Int. J. Dev. Biol. (2003) 47: 123-133

Charting the course of ovarian development in vertebrates.
Kelly A Loffler and Peter Koopman
Int. J. Dev. Biol. (2002) 46: 503-510

mgm 1, the earliest sex-specific germline marker in Drosophila, reflects expression of the gene esg in male stem cells.
Adrian Streit, Luca Bernasconi, Pavel Sergeev, Alex Cruz and Monica Steinmann-Zwicky
Int. J. Dev. Biol. (2002) 46: 159-166

Musca domestica, a window on the evolution of sex-determining mechanisms in insects.
Andreas Dübendorfer, Monika Hediger, Géza Burghardt and Daniel Bopp
Int. J. Dev. Biol. (2002) 46: 75-79

Sexual differentiation of the somatic gonad tissue in marine bivalve mollusks: esterase- and fibronectin-like recognition signals
M Paz, A Mikhailov, M Torrado
Int. J. Dev. Biol. (2001) 45: S119-S120

Sex reversal of the newt Triturus cristatus reared at extreme temperatures.
H Wallace and B M Wallace
Int. J. Dev. Biol. (2000) 44: 807-810

2006 ISI **Impact Factor = 3.577**